REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	28.Jun.06	THESIS

4. TITLE AND SUBTITLE
ENDOSULFAN-ALPHA INDUCES CYP2B6 AND CYP3A4 BY ACTIVATING THE PREGNANE X RECEPTOR BUT NOT THE CONSTITUTIVE ANDROSTANE RECEPTOR.

5. FUNDING NUMBERS

6. AUTHOR(S)
CAPT CASABAR RICHARD C

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
NORTH CAROLINA STATE UNIVERSITY

8. PERFORMING ORGANIZATION REPORT NUMBER
CI04-1805

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
THE DEPARTMENT OF THE AIR FORCE
AFIT/CIA, BLDG 125
2950 P STREET
WPAFB OH 45433

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Unlimited distribution
In Accordance With AFI 35-205/AFIT Sup

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS	15. NUMBER OF PAGES
	83
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

ENDOSULFAN-ALPHA INDUCES CYP2B6 AND CYP3A4 BY ACTIVATING THE PREGNANE X RECEPTOR BUT NOT THE CONSTITUTIVE ANDROSTANE RECEPTOR

by

RICHARD CHRISTOPHER TOMAS CASABAR

A thesis submitted to the Graduate Faculty of

North Carolina State University

In partial fulfillment of the

Requirements for the degree of

Master of Science

In

TOXICOLOGY

Raleigh, NC

2006

Approved by:

ERNEST HODGSON

ANDREW D. WALLACE
Co-chair of Advisory Committee

RANDY L. ROSE
Co-chair of Advisory Committee

THE VIEWS EXPRESSED IN THIS ARTICLE ARE THOSE OF THE AUTHOR AND DO NOT REFLECT THE OFFICIAL POLICY OR POSITION OF THE UNITED STATES AIR FORCE, DEPARTMENT OF DEFENSE, OR THE U.S. GOVERNMENT.

ABSTRACT

CASABAR, RICHARD CHRISTOPHER TOMAS. Endosulfan-alpha Induces CYP2B6 and CYP3A4 via the Pregnane X Receptor but not the Constitutive Androstane Receptor. (Under the directions of Randy L. Rose and Andrew D. Wallace.)

The purpose of this research was to establish the metabolic pathway of endosulfan in humans and to elucidate a potential mechanism for endosulfan's endocrine disruptive effects. We hypothesized that endosulfan may exert its endocrine disrupting effects by activating the pregnane X receptor (PXR) and/or the constitutive androstane receptor (CAR) and inducing the expression levels of cytochrome P450 (CYP) enzymes, thereby increasing metabolic rates and the biotransformation of testosterone. In these studies, we utilized endosulfan-α, the more predominant isomer in technical-grade endosulfan.

In Chapter 1, we determined that endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, in pooled human liver microsomes (K_m = 9.8 μM, V_{max} = 178.5 pmol/mg/min). With the use of recombinant cytochrome P450 (rCYP) isoforms expressed in baculovirus-infected cells (supersomes), we identified CYP2B6 (K_m = 16.2 μM, V_{max} = 11.4 nmol/nmol CYP/min) and CYP3A4 (K_m = 14.4 μM, V_{max} = 1.3 nmol/nmol CYP/min) as the primary enzymes catalyzing the metabolism of endosulfan-α, albeit CYP2B6 had an 8-fold higher intrinsic clearance rate (CL_{int} = 0.70 μL/min/pmol CYP) than CYP3A4 (CL_{int} = 0.09 μL/min/pmol CYP). Using commercially available individual human liver microsomes (HLM), a strong correlation was observed with endosulfan sulfate formation and S-mephenytoin N-demethylase activity of CYP2B6 (r^2 = 0.79) and a moderate correlation with testosterone 6-β-hyroxylase activity of CYP3A4 (r^2 = 0.54). Ticlopidine (5 μM), a potent mechanism-based inhibitor of CYP2B6, and ketoconazole (10 μM), a selective CYP3A4

inhibitor, together inhibited approximately 90% of endosulfan sulfate formation in HLMs, presenting the possibility of utilizing endosulfan-α as a simultaneous *in vitro* probe for CYP2B6 and CYP3A4 activity. The percent total normalized rate (% TNR) was calculated to estimate the contribution of each rCYP in the total metabolism of endosulfan-α in HLMs and was used to validate the percent inhibition (% I) by ticlopidine and ketoconazole in this study. Five of the six HLMs in this study showed a good correlation between the % I with ticlopidine and ketoconazole in the same incubation and the combined % TNRs for CYP2B6 and CYP3A4.

In Chapter 2, we investigated if endosulfan-α induces CYP3A4 and CYP2B6 by activating PXR and/or CAR. These interactions were explored by transient transfection assays in the HepG2 cell line using CYP3A4-luciferase, CYP2B6-luciferase, human PXR (hPXR), and mouse CAR (mCAR) plasmids. Endosulfan-α (10 μM) treatments resulted in 11-fold induction of CYP3A4 and 16-fold induction of CYP2B6 promoter activities over untreated controls in the presence of hPXR. The metabolite endosulfan sulfate (10 μM) also induced CYP3A4 and CYP2B6 promoter activities by 6-fold and 12-fold, respectively. In the presence of mCAR and the transcriptional repressor androstenol, endosulfan-α only reversed androstenol repression weakly and induced CYP2B6 promoter activity by 3-fold over control. Utilizing S9 samples from primary human hepatocytes for western blotting, it was determined that endosulfan-α induced CYP2B6 translation in a dose-dependent manner (0.1 to 50 μM) and CYP3A4 translation, but only at 50 μM dose. Using the same S9 samples for testosterone (TST) metabolism assays, an increase in TST metabolites was observed, albeit only at 50 μM endosulfan-α treatment.

This research has determined that endosulfan-α is metabolized by HLMs to a single metabolite, endosulfan sulfate, and that it can be utilized to simultaneously probe for CYP2B6 and 3A4 catalytic activities. These studies have also demonstrated that endosulfan-α activates hPXR, but only weakly as a ligand for mCAR, and induces CYP2B6 and CYP3A4 promoter activity and protein expression, and that it may affect endocrine homeostasis by inducing P450s.

ABSTRACT

CASABAR, RICHARD CHRISTOPHER TOMAS. Endosulfan-alpha Induces CYP2B6 and CYP3A4 via the Pregnane X Receptor but not the Constitutive Androstane Receptor. (Under the directions of Randy L. Rose and Andrew D. Wallace.)

The purpose of this research was to establish the metabolic pathway of endosulfan in humans and to elucidate a potential mechanism for endosulfan's endocrine disruptive effects. We hypothesized that endosulfan may exert its endocrine disrupting effects by activating the pregnane X receptor (PXR) and/or the constitutive androstane receptor (CAR) and inducing the expression levels of cytochrome P450 (CYP) enzymes, thereby increasing metabolic rates and the biotransformation of testosterone. In these studies, we utilized endosulfan-α, the more predominant isomer in technical-grade endosulfan.

In Chapter 1, we determined that endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, in pooled human liver microsomes (K_m = 9.8 μM, V_{max} = 178.5 pmol/mg/min). With the use of recombinant cytochrome P450 (rCYP) isoforms expressed in baculovirus-infected cells (supersomes), we identified CYP2B6 (K_m = 16.2 μM, V_{max} = 11.4 nmol/nmol CYP/min) and CYP3A4 (K_m = 14.4 μM, V_{max} = 1.3 nmol/nmol CYP/min) as the primary enzymes catalyzing the metabolism of endosulfan-α, albeit CYP2B6 had an 8-fold higher intrinsic clearance rate (CL_{int} = 0.70 μL/min/pmol CYP) than CYP3A4 (CL_{int} = 0.09 μL/min/pmol CYP). Using commercially available individual human liver microsomes (HLM), a strong correlation was observed with endosulfan sulfate formation and S-mephenytoin N-demethylase activity of CYP2B6 (r^2 = 0.79) and a moderate correlation with testosterone 6-β-hyroxylase activity of CYP3A4 (r^2 = 0.54). Ticlopidine (5 μM), a potent mechanism-based inhibitor of CYP2B6, and ketoconazole (10 μM), a selective CYP3A4

inhibitor, together inhibited approximately 90% of endosulfan sulfate formation in HLMs, presenting the possibility of utilizing endosulfan-α as a simultaneous *in vitro* probe for CYP2B6 and CYP3A4 activity. The percent total normalized rate (% TNR) was calculated to estimate the contribution of each rCYP in the total metabolism of endosulfan-α in HLMs and was used to validate the percent inhibition (% I) by ticlopidine and ketoconazole in this study. Five of the six HLMs in this study showed a good correlation between the % I with ticlopidine and ketoconazole in the same incubation and the combined % TNRs for CYP2B6 and CYP3A4.

In Chapter 2, we investigated if endosulfan-α induces CYP3A4 and CYP2B6 by activating PXR and/or CAR. These interactions were explored by transient transfection assays in the HepG2 cell line using CYP3A4-luciferase, CYP2B6-luciferase, human PXR (hPXR), and mouse CAR (mCAR) plasmids. Endosulfan-α (10 μM) treatments resulted in 11-fold induction of CYP3A4 and 16-fold induction of CYP2B6 promoter activities over untreated controls in the presence of hPXR. The metabolite endosulfan sulfate (10 μM) also induced CYP3A4 and CYP2B6 promoter activities by 6-fold and 12-fold, respectively. In the presence of mCAR and the transcriptional repressor androstenol, endosulfan-α only reversed androstenol repression weakly and induced CYP2B6 promoter activity by 3-fold over control. Utilizing S9 samples from primary human hepatocytes for western blotting, it was determined that endosulfan-α induced CYP2B6 translation in a dose-dependent manner (0.1 to 50 μM) and CYP3A4 translation, but only at 50 μM dose. Using the same S9 samples for testosterone (TST) metabolism assays, an increase in TST metabolites was observed, albeit only at 50 μM endosulfan-α treatment.

This research has determined that endosulfan-α is metabolized by HLMs to a single metabolite, endosulfan sulfate, and that it can be utilized to simultaneously probe for CYP2B6 and 3A4 catalytic activities. These studies have also demonstrated that endosulfan-α activates hPXR, but only weakly as a ligand for mCAR, and induces CYP2B6 and CYP3A4 promoter activity and protein expression, and that it may affect endocrine homeostasis by inducing P450s.

DEDICATION

I dedicate this work to my lovely wife and to the one who makes us smile every day, our son.

I also would like to dedicate this thesis to a friend and mentor, the late Dr. Randy L. Rose, whose guidance have been very instrumental in the completion of my research and thesis writing.

BIOGRAPHY

Richard Casabar is a Captain in the United States Air Force (USAF). He is a laboratory officer and was previously assigned to Andersen AFB, Guam and Tinker AFB, Oklahoma. Richard was born in Baguio City, Philippines. He graduated from St. Philomena's Academy High School, Pozorrubio, Pangasinan, Philippines in 1988. He attended the Medical Technology department at the University of Santo Tomas, Manila, Philippines in 1988-1990. He immigrated to the United States on December 1990 and went on to complete his Bachelor of Science in Medical Technology from the University of Hawaii at Manoa in 1995. He was directly commissioned as a second lieutenant in the USAF in 1998. Richard is a certified Medical Technologist by the American Society for Clinical Pathology.

ACKNOWLEDGMENTS

I would like to thank Dr. Parik Das, Yan Cao, Amin Usmani, Leslie Tompkins, Beth Cooper, Jae Young Kim, Rakesh Ranjan, and Eddie Croom for their invaluable help and contributions to this work.

I would like to give special thanks to my advisers and graduate committee members: Dr. Andrew Wallace, Dr. Ernest Hodgson, and the late Dr. Randy L. Rose.

Most importantly, I would like to thank my wife and son for their love and support, which inspired me to complete this work. And lastly, I owe a great gratitude to my uncle [redacted] and my late grandma Ninay for teaching me the values which have continued to guide me in life and in my career.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

Chapter 1

Chapter 2

Page

Chapter 2

INTRODUCTION

1. Endosulfan background and history.

Endosulfan is an organochlorine pesticide which is continually used around the world for applications on vegetables, fruits, and non-food crops such as cotton and tobacco (USEPA, 2002). It is also found as a contaminant in soil and groundwater at toxic superfund sites in the United States (USEPA). Technical grade endosulfan is sold under the tradename of Thiodan®, which is a mixture of 70% α and 30% β isomers (ATSDR, 2000).

In 1954, endosulfan (Thiodan®) was first introduced in the United States by Farbwerke Hoechst AG (Maier-Bode, 1968). Endosulfan production in the US in 1974 was around 3 million pounds per year (Sittig, 1980). The major US manufacturer of endosulfan was FMC Corporation. It is no longer manufactured in the US and production ceased in 1982 (ATSDR, 2000). In 1984, the worldwide production of endosulfan was estimated at 10,000 metric tons (WHO, 1984). There are no recent reports on current estimates of worldwide production. At present, endosulfan is still produced in Germany, Great Britain, India, Israel, Italy, Mexico, and Taiwan (ATSDR 2000).

2. Endosulfan is a hazardous chemical.

2.1. Toxicity.

The central nervous system is the major target of endosulfan toxicity in humans and animals. It can also cause hematological effects and nephrotoxicity (ATSDR, 2000). In rodent studies, endosulfan-α is 3 times more toxic than endosulfan-β (Dorough et al, 1978). The US Environmental Protection Agency (USEPA) classifies endosulfan as Category Ib –

Highly Hazardous. Excessive and improper application and handling of endosulfan have been linked to congenital physical disorders, mental retardations and deaths in farm workers and villagers in developing countries in Africa, southern Asia and Latin America (Environmental Justice Foundation, 2002).

2.1.1. Acute toxicity.

Endosulfan is highly toxic to mammals, with an acute oral LD_{50} (with single exposure) for rats of 24 mg/kg (Boyd et al., 1970). In mice (males), the LD_{50} of endosulfan with single exposure is 7.4 mg/kg (Gupta et al., 1981). The LD_{50} of endosulfan-α in mice (females) with single exposure is 14 mg/kg (ATSDR, 2000). In humans (males), 260 mg/kg of endosulfan (technical-grade) was lethal in one case and in another case have caused convulsions, cerebral edema, cerebral herniation, and sustained epileptic state (Boereboom et al., 1998).

Acute exposure to huge amounts of endosulfan results in hyperactivity, muscle tremors, ataxia, and hyperactivity (ATSDR, 2000). The two reported mechanisms of neuro-toxicity include: 1) blockage of neurotransmitter receptors (Zaidi et al., 1985; Abalis et al., 1986) and 2) interference with synthesis, degradation, and release/re-uptake of neurotransmitters (Paul et al., 1994). GABA-antagonism is the widely accepted mechanism of acute or neuron-toxicity (ATSDR, 2000).

In the US, endosulfan was linked to the death of one farmer and permanent neurological impairment of another (Brandt et al., 2001). In Cuba, 15 people died of endosulfan poisoning in the province of Matanzas, where a total of 63 people became ill after eating food contaminated with endosulfan (Campaigner, Aug 1999). In Borgou, Benin, endosulfan caused 37 deaths during the 1999-2000 cotton season (Ton et al., 2000). In the same region, a boy died in 1999 after ingesting corn contaminated with endosulfan (Myers, 2000). In

Sudan in 1988, endosulfan barrels washed in irrigation canals caused fish kills and death of three people who drank water from the canal (Dinham, 1993). In 1993 in Columbia, 60 poisonings and 1 death occurred due to endosulfan use on coffee (PANUPS, 1994).

2.1.2. Chronic toxicity.

The chronic sub-lethal effects of endosulfan that were manifested in laboratory animals include liver and kidney toxicity, hematological effects, interference with the immune system, and alterations in reproductive organs of males. In male children chronically exposed to endosulfan from aerial-spraying of cashew plantations in north India, a delay in sexual maturity was observed (Saiyed et al., 2003). Studies in humans and animals have provided no evidence of carcinogenicity for endosulfan (ATSDR, 2000).

2.2. Persistence.

Endosulfan is recognized as a Persistent Toxic Substance (PTS) by the United Nations Environment Programme (UNEP, 2003). In soil, endosulfan can persist for a long period with half lives of 60 and 800 days for endosulfan-α and β, respectively (Stewart and Cairns, 1974), but is rapidly degraded in water (ATSDR, 2000).

2.3. Bioaccumulation.

The maximum bioconcentration factors (BCF) of endosulfan in aquatic systems are usually less than 3,000, and metabolism studies with laboratory animals indicate that endosulfan does not bioconcentrate in fatty tissues and milk (ATSDR, 2000), suggesting that it has only a moderate potential for bioaccumulation.

3. Potential for human exposure to endosulfan.

Endosulfan contamination is found throughout the environment and is present in many superfund sites in the United States (Kullman and Matsumura, 1996; ATSDR, 2000). A review of commonly used pesticides in Canadian air claimed that endosulfan is one of the most abundant and ubiquitous organochlorine pesticides in the North American atmosphere today (Tuduri et al, 2006). A study in Denmark on pesticide residues (including endosulfan) in apples reported mean concentrations of 0.482, 0.468, and 0.024 mg/kg for endosulfa-α, β, and sulfate, respectively (Rasmusssen et al., 2003). The study added that simple washing does not significantly decrease the amount of pesticides and that endosulfan sulfate residues were increased by 34% during long-term storage of apples.

Humans can be exposed to endosulfan percutaneously or through ingestion and inhalation (Kalender et al., 2004). For individuals in occupational settings or people living near hazardous waste sites or agricultural fields applied with endosulfan, the primary routes of exposure would be inhalation and dermal exposure. In a study of spraymen's exposure to endosulfan from application of this pesticide on fruit orchards, a mean dermal exposure 24.7 mg/hr (range of 0.6-95.3 mg/hr) and a mean inhalation exposure of 0.02 ng/hr (range of 0.01-0.05 mg/hr) were estimated (Wolfe et al., 1972). In another study of women living in Southern Spain, where large amounts of endosulfan were used in intensive greenhouse agriculture, endosulfan residues were detected in breast milk (11.38 mg/mL) and adipose tissues (17.72 ng/g) of these women (Cerrillo et al., 2005). Cerrillo et al further argued that their findings support the lipophilicity of endosulfan and its elimination by milk secretion. Endosulfan and metabolites were also detected in fats of 30-40% of children hospitalized after exposure to endosulfan in agricultural regions of Spain (Olea et al., 1999).

For the general population, the main route of exposure to endosulfan is ingestion of food or tobacco products containing residues of endosulfan. In 2001, the Total Diet Study conducted by the FDA's pesticide residue monitoring program determined endosulfan as one of the 19 most frequently found residues (those found in >2% of the samples), endosulfan as one of the 5 most frequently observed chemicals, and endosulfan with an 18 percent occurrence in the four market baskets analyzed (1030 food items). Albeit, the levels of endosulfan residues, were well below regulatory limits (FDA, 2001).

3.1. Release of endosulfan to the environment.

The major source of endosulfan distribution to the environment is spray drift from aerial applications of this pesticide on crops. However, endosulfan can also be distributed through run-off water from fields sprayed with this pesticide (Kennedy et al, 2001) and through volatilization where it can undergo long-range atmospheric transport, by which traces of this pesticide have been found in the Arctic (Jantunen and Bidleman, 1998). Endosulfan has been detected in air, surface water, groundwater, soil, and sediment (ATSDR, 2000).

3.2. Environmental fate.

Endosulfan is not very soluble in water. The log K_{OW} (Octanol-Water partition coefficient - describes the partitioning of organic pollutants between water phase and octanol) of endosulfan-α and β are 3.83 and 3.52, respectively (Hansch et al., 1995). Endosulfan has been found at low concentrations in few surface water and groundwater samples at hazardous waste sites (ATSDR, 2000).

Endosulfan partitions to the atmosphere and soils or sediments (ATSDR, 2000). The Henry's law constant (describes partitioning of organic pollutants between the water phase and air in the environment) for endosulfan-α and β are 0.7-12.9 and 0.04-2.12 Pa m^3 mol^{-1} at

20°C, respectively (German Federal Environment Agency, 2004). These values indicate that the α-isomer is expected to be more mobile in the atmosphere because it is more volatile than the β isomer. This is supported by a review on currently used pesticides in Canadian air which stated that endosulfan-β is always detected in lesser amounts than the α-isomer in the atmosphere, partly due to the composition of technical-endosulfan (contains more α-isomer) but also because the α-isomer is more volatile (Tuduri et al., 2006).

Results of several studies indicate that both isomers of endosulfan strongly adsorb to soil. The K_{OC} (organic carbon-water partition coefficient - describes partitioning of organic pollutants between water phase and natural organic matter in soils or sediments) of endosulfan-α and β are estimated to be 2,887 and 1,958, respectively, suggesting that mobility of endosulfan in soil or sediment is expected to be slight (ATSDR, 2000).

Endosulfan does not bioaccumulate to high concentrations in terrestrial or aquatic organisms. Maximum bioconcentration factors are less than 3,000, and residues are eliminated from organisms very rapidly (ATSDR, 2000).

3.3. Degradation in the environment.

Endosulfan-β is slowly converted to the more stable α-isomer at high temperatures (Rice et al., 1997). Endosulfan is degraded to endosulfan diol by hydrolysis in surface water and groundwater. In sediment and soil, endosulfan is degraded primarily to endosulfan sulfate by fungal metabolism and to endosulfan diol by bacterial metabolism. Biotransformation of endosulfan in soil under aerobic conditions yield endosulfan sulfate, endosulfan diol, and endosulfan lactone. In anaerobic conditions, the metabolites endosulfan diol, endosulfan sulfate, and endosulfan hydroxyether are formed (ATSDR, 2000).

3.4 Metabolism in mammals.

In animals, endosulfan-α or β can be converted to endosulfan sulfate or endosulfan diol (WHO, 1984), which can be further metabolized to endosulfan lactone, hydroxyether, and ether (ATSDR, 2000). In humans, the metabolites endosulfan sulfate, alcohol, ether, and lactone were detected in urine (Martinez Vidal et al., 1998; ATSDR, 2000) and serum (Arrebola et al., 2001).

Although the metabolites diol, ether, and lactone are non-toxic, the metabolite endosulfan sulfate is nearly as toxic as the parent endosulfan, does not undergo further degradation, and residues tend to increase in the environment (Kennedy et al., 2001).

4. Research goal and hypothesis.

The purpose of the present research project is to elucidate a potential mechanism of endosulfan's endocrine disruptive effects. We hypothesize that endosulfan may exert its endocrine disrupting effects by activating the pregnane X receptor (PXR) and/or the constitutive androstane receptor (CAR) and inducing the expression levels of cytochrome P450 (CYP) enzymes, thereby increasing metabolic rates and the biotransformation of testosterone.

5. Potential mechanisms for endocrine disrupting effects of endosulfan.

Endocrine disrupting chemicals (EDCs) show their effects after a short term and at low levels of exposure, and affect endocrine signaling to a variety of organs which results in inhibited growth and reproductive development (LeBlanc, 2005). The following are potential mechanisms for the endocrine disrupting effects of endosulfan.

5.1. Interactions with steroid hormone receptors.

Endocrine disruption can occur when xenobiotics mimic endogenous steroid hormones. Endosulfan has been shown to act as an estrogen agonist, triggering the formation of increased numbers of alveolar buds and telomerase reverse transcriptase (TERT) mRNA expression in mouse mammary glands (Je et al., 2005). In another *in vitro* study, endosulfan has shown estrogenic effects on human estrogen sensitive cells (Soto et al., 1994). In contrary, endosulfan did not show estrogenic response in ovariectomized mice, and results of combined endosulfan and estradiol treatments indicated no estrogenic nor anti-estrogenic effects of endosulfan (Hiremath and Kaliwal, 2003). Another *in vivo* study reported that uterine weight in female mice was not affected by endosulfan, whereas estradiol gave a strong positive response (Shelby et al., 1996). The ATSDR stated that studies *in vivo* indicate endosulfan is neither estrogenic nor disruptive of thyroid or pituitary hormone levels in females, despite of its weak estrogenic effects in *in vitro* systems (ATSDR, 2000).

5.2. Alteration of gene expression or activity of enzymes involved in the synthesis of steroids.

Another mechanism for endocrine disruption is for xenobiotics to stimulate enzymes involved in the synthesis of steroids (Hilscherova et al., 2004). In rats, after sub-chronic exposures to endosulfan, a profound decrease in levels of plasma gonadotrophins and testosterone, testicular testosterone, and a considerable decrease in activities of enzymes of androgen biosynthesis (3 beta- and 17 beta-hydroxysteroid dehydrogenases) were seen (Singh and Pandey, 1990). In a study in amphibians, it was suggested that endosulfan has the capacity to impair secretory capacity of adrenal cells, albeit the LC50:EC50 ratio was low (LC50 – concentration that kills 50% of steroidogenic cells and EC50 – concentration that

impairs corticosterone synthesis; the higher the ratio, the higher the potential for endocrine disruption) (Goulet and Hontela, 2003).

5.3. Induction of steroid metabolizing enzymes.

A third mechanism for endocrine disruption is via xenobiotic induction of hepatic steroid metabolizing enzymes, resulting in increased metabolism and inactivation of steroids (You, 2004). Endosulfan has been shown to increase rodent liver weights and elevate microsomal enzyme levels (Gupta and Gupta, 1977). In mice, endosulfan treatments increased testosterone metabolism and clearance, and this study further suggested that endosulfan may have selectively induced CYP activities most indicative of the phenobarbital (PB)-type response (Wilson and LeBlanc, 1998). In male rats exposed to endosulfan, a dose related decrease in testosterone was seen (Singh and Pandey, 1990). Another study in rats demonstrated that endosulfan induced P450s in hepatic and extra-hepatic tissues (Siddiqui et al., 1987). A study utilizing transient transfection of the human pregnane X receptor (hPXR) expression vector and CYP3A4 luciferase reporter vector into human hepatoma cell line HepG2 showed that endosulfan induced the CYP3A4 promoter activity by activating the hPXR (Coumoul et al., 2002).

6. Why study endosulfan?

Endocrine disruption seen in children in developing countries living nearby fields sprayed with endosulfan could have been caused by chronic exposure to this pesticide. In the US, a concern for chronic exposure of children to endosulfan should be raised for the following reasons: 1) endosulfan is a pesticide still commonly applied to fruits and vegetables and its residues are detected in a number of food items from supermarkets, albeit at levels below

regulatory limits; 2) endosulfan is a common contaminant found in the environment; 3) endosulfan and its metabolite endosulfan sulfate are persistent substances; and 4) children are particularly susceptible to the toxicity of endosulfan (ATSDR, 2000).

The results of the Saiyed et al, 2003 epidemiological study, where testosterone levels were decreased and delayed male sexual maturity were seen in children who were chronically exposed to endosulfan, coupled with evidence that organochorines induce CYP enzymes (Siddiqui et al., 1987), led us to think that a very likely mechanism for endosulfan's endocrine disruption is through its interaction with PXR and/or CAR and induction of the CYP enzymes.

This research consisted of two major areas of investigation. First, the human metabolic pathway of endosulfan was determined and is discussed in Chapter 1. Second, these studies characterized the role of endosulfan in inducing P450s as a possible mechanism of endocrine disruption and are discussed in Chapter 2.

References

Abalis IM, Eldefrawi ME and Eldefrawi AT (1986) Effects of insecticides on GABA-induced chloride influx into rat brain microsacs. *J Toxicol Environ Health* **18:**13-23.

Arrebola FJ, Martinez Vidal JL and Fernandez-Gutierrez A (2001) Analysis of endosulfan and its metabolites in human serum using gas chromatography-tandem mass spectrometry. *J Chromatogr Sci* **39:**177-182.

ATSDR (2000) Toxicological Profile for Endosulfan, Agency for Toxic Substances and Disease Registry (ATSDR), U.S. Department of Health and Human Services.

Boereboom FT, van Dijk A, van Zoonen P and Meulenbelt J (1998) Nonaccidental endosulfan intoxication: a case report with toxicokinetic calculations and tissue concentrations. *J Toxicol Clin Toxicol* **36:**345-352.

Boyd EM, Dobos I and Krijnen CJ (1970) Endosulfan toxicity and dietary protein. *Arch Environ Health* **21:**15-19.

Brandt VA, Moon S, Ehlers J, Methner MM and Struttmann T (2001) Exposure to endosulfan in farmers: two case studies. *Am J Ind Med* **39:**643-649.

Cerrillo I, Granada A, Lopez-Espinosa MJ, Olmos B, Jimenez M, Cano A, Olea N and Fatima Olea-Serrano M (2005) Endosulfan and its metabolites in fertile women, placenta, cord blood, and human milk. *Environ Res* **98:**233-239.

Coumoul X, Diry M and Barouki R (2002) PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. *Biochem Pharmacol* **64:**1513-1519.

Dinham B (1993) *The Pesticide Hazard. A Global Health and Environmental Audit.* Zed Books, London.

Environmental Justice Foundation (2002), End of the Road for Endosulfan, http://www.ejfoundation.org/pdfs/end_of_the_road.pdf

FDA (2001) Total Diet Study, Pesticide Residue Monitoring 2001, Food and Drug Administration, http://www.cfsan.fda.gov

German Federal Environment Agency (2004) Draft Dosier Endosulfan, September 2004, Umweltbundesamt, Berlin.

Global Pesticide Campaigner (Aug 1999) Pesticide poisoning in Cuba, in: *Pesticide Action Network North America*, pp 28, Pesticide Action Network North America, San Francisco, CA.

Goulet BN and Hontela A (2003) Toxicity of cadmium, endosulfan, and atrazine in adrenal steroidogenic cells of two amphibian species, Xenopus laevis and Rana catesbeiana. *Environ Toxicol Chem* **22:**2106-2113.

Gupta PK and Gupta RC (1977) Effect of endosulfan pretreatment on organ weights and on pentobarbital hypnosis in rats. *Toxicology* **7:**283-288.

Gupta PK, Murthy RC and Chandra SV (1981) Toxicity of endosulfan and manganese chloride: cumulative toxicity rating. *Toxicol Lett* **7:**221-227.

Hansch C, Leo A and Hoekman D (1995) *Exploring QSAR. Hydrophobic, electronic, and steric constants.* American Chemical Society, Washington, DC.

Hilscherova K, Jones P, Gracia T, Newsted J, Zhang X, Sanderson JT, Yu R, Wu R and Giesy J (2004) Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. *Tox Sci* 81:78-89

Hiremath MB and Kaliwal BB (2003) Evaluation of estrogenic activity and effect of endosulfan on biochemical constituents in ovariectomized (OVX) Swiss albino mice. *Bull Environ Contam Toxicol* **71:**458-464.

Jantunen LM and Bidleman TF (1998) Organochlorine pesticides and enantiomers of chiral pesticides in Arctic Ocean water. *Arch Environ Contam Toxicol* **35:**218-228.

Je KH, Kim KN, Nam KW, Cho MH and Mar W (2005) TERT mRNA expression is up-regulated in MCF-7 cells and a mouse mammary organ culture (MMOC) system by endosulfan treatment. *Arch Pharm Res* **28:**351-357.

Kalender S, Kalender Y, Ogutcu A, Uzunhisarcikli M, Durak D and Acikgoz F (2004) Endosulfan-induced cardiotoxicity and free radical metabolism in rats: the protective effect of vitamin E. *Toxicology* **202:**227-235.

Kennedy IR, Sanchez-Bayo F, Kimber SW, Hugo L and Ahmad N (2001) Off-site movement of endosulfan from irrigated cotton in New South Wales. *J Environ Qual* **30:**683-696.

Kullman SW and Matsumura F (1996) Metabolic pathways utilized by Phanerochaete chrysosporium for degradation of the cyclodiene pesticide endosulfan. *Appl Environ Microbiol* **62:**593-600.

LeBlanc G (2005) Lecture on Environmental Signalling and Endocrine Disruption, Tox 715 Environmental Toxicology course (Fall 2005), North Carolina State Univ., Raleigh, NC.

Maier-Bode H (1968) Properties, effect, residues and analytics of the insecticide endosulfan. *Residue Rev* **22:**1-44.

Martinez Vidal JL, Arrebola FJ, Fernandez-Gutierrez A and Rams MA (1998) Determination of endosulfan and its metabolites in human urine using gas chromatography-tandem mass spectrometry. *J Chromatogr B Biomed Sci Appl* **719:**71-78.

Myers D (2000) Cotton Tales. *New Internationalist.*

Olea N, Olea-Serrano F, Lardelli-Claret P, Rivas A and Barba-Navarro A (1999) Inadvertent exposure to xenoestrogens in children. *Toxicology and Industrial Health* **15:**151-158.

PANUPS (1994) International Citizen's Campaign Target Hoechst Pesticides.

Paul V, Balasubramaniam E and Kazi M (1994) The neurobehavioural toxicity of endosulfan in rats: a serotonergic involvement in learning impairment. *Eur J Pharmacol* **270:**1-7.

Rasmusssen RR, Poulsen ME and Hansen HCB (2003) Distribution of multiple pesticide residues in apple segments after home processing. *Food Additives and Contaminants* **20:**1044-1063.

Rice CP, Hapeman CJ and Chernyak SM (1997) Experimental evidence for the interconversion of endosulfan isomers. *Abstracts of Papers of the American Chemical Society* **213:**224-ENVR.

Saiyed H, Dewan A, Bhatnagar V, Shenoy U, Shenoy R, Rajmohan H, Patel K, Kashyap R, Kulkarni P, Rajan B and Lakkad B (2003) Effect of endosulfan on male reproductive development. *Environ Health Perspect* **111:**1958-1962.

Shelby MD, Newbold RR, Tully DB, Chae K and Davis VL (1996) Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. *Environ Health Perspect* **104:**1296-1300.

Siddiqui MKJ, Anjum F and Qadri SSH (1987) Some Metabolic Changes Induced by Endosulfan in Hepatic and Extra Hepatic Tissues of Rat. *Journal of Environmental*

Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes **22:**553-564.

Singh SK and Pandey RS (1990) Effect of Subchronic Endosulfan Exposures on Plasma Gonadotropins, Testosterone, Testicular Testosterone and Enzymes of Androgen Biosynthesis in Rat. *Indian Journal of Experimental Biology* **28:**953-956.

Sittig M (1980) *Priority Toxic Pollutants: Health impacts and allowable limits.* Noyes Data Corp, Park Ridge, NJ.

Soto AM, Chung KL and Sonnenschein C (1994) The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ Health Perspect* **102:**380-383.

Stewart DKR and Cairns KG (1974) Endosulfan Persistence in Soil and Uptake by Potato-Tubers. *Journal of Agricultural and Food Chemistry* **22:**984-986.

Ton P, Tovignan S and Vodouche S (Mar 2000) Endosulfan deaths and Poisonings in Benin (47 PNN ed, pp 12-14, Pesticide Action Network UK.

Tuduri L, Harner T, Blanchard P, Li YF, Poissant L, Waite DT, Murphy C and Belzer W (2006) A review of currently used pesticides (CUPs) in Canadian air and precipitation: Part 1: Lindane and endosulfans. *Atmospheric Environment* **40:**1563-1578.

UNEP (2003) State of the Environment, Governing Council of the United Nations Environment Programme.

USEPA U.S. Environmental Protection Agency, Superfund Information Systems, Comprehensive Environmental Response, Compensation and Liability Information System (CERCLIS) Database.

USEPA (2002) Reregistration Eligibility Decision (R.E.D) for Endosulfan, U.S. Environmental Protection Agency (USEPA).

WHO (1984) Endosulfan. International Programme on Chemical Safety. Environmental Health Criteria 40., pp 1-62, World Health Organization, Geneva, Switzerland.

Wilson VS and LeBlanc GA (1998) Endosulfan elevates testosterone biotransformation and clearance in CD-1 mice. *Toxicol Appl Pharmacol* **148:**158-168.

Wolfe HR, Staiff C, Armstron.Jf and Comer SW (1972) Exposure of Spraymen to Pesticides. *Archives of Environmental Health* **25:**29-&.

You L (2004) Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chemico-Biological Interactions* **147:**233-246.

Zaidi NF, Agrawal AK, Anand M and Seth PK (1985) Neonatal endosulfan neurotoxicity: behavioral and biochemical changes in rat pups. *Neurobehav Toxicol Teratol* **7:**439-442.

CHAPTER 1

METABOLISM OF ENDOSULFAN-ALPHA BY HUMAN LIVER MICROSOMES AND ITS UTILITY AS A SIMULTANEOUS *IN VITRO* PROBE FOR CYP2B6 AND CYP3A4

Richard C.T. Casabar, Andrew D. Wallace, Ernest Hodgson, and Randy L. Rose

Department of Environmental and Molecular Toxicology, Box 7633, North Carolina State University, Raleigh, NC 27695

*This manuscript was submitted to *Drug Metabolism and Disposition* and is formatted according to the journal's requirements.

ENDOSULFAN METABOLISM AND POTENTIAL AS CYP2B6 AND 3A4 PROBE

Corresponding Author: Randy L. Rose
Department of Environmental and Molecular Toxicology,
Box 7633, North Carolina State University, Raleigh, NC 27695
919-515-4378, (Fax) 919-515-7169, randy_rose@ncsu.edu

Number of text pages: 19

Number of Tables: 5

Number of Figures: 5

Number of References: 26

Number of words in Abstract: 240

Number of words in Introduction: 461

Number of words in Discussion: 851

Non-standard abbreviations used: Cytochrome P450 (P450 or CYP); human liver microsomes (HLM); percent total normalized rate (% TNR); percent inhibition (% I); acetonitrile (ACN); flavin-containing monooxygenase (FMO).

METABOLISM OF ENDOSULFAN-ALPHA BY HUMAN LIVER MICROSOMES AND ITS UTILITY AS A SIMULTANEOUS *IN VITRO* PROBE FOR CYP2B6 AND CYP3A4

ABSTRACT

Endosulfan-α was metabolized to a single metabolite, endosulfan sulfate, in pooled human liver microsomes (K_m = 9.8 μM, V_{max} = 178.5 pmol/mg/min). With the use of recombinant cytochrome P450 (rCYP) isoforms, we identified CYP2B6 (K_m = 16.2 μM, V_{max} = 11.4 nmol/nmol CYP/min) and CYP3A4 (K_m = 14.4 μM, V_{max} = 1.3 nmol/nmol CYP/min) as the primary enzymes catalyzing the metabolism of endosulfan-α, albeit CYP2B6 had an 8-fold higher intrinsic clearance rate (CL_{int} = 0.70 μL/min/pmol CYP) than CYP3A4 (CL_{int} = 0.09 μL/min/pmol CYP). Using 16 individual human liver microsomes (HLM), a strong correlation was observed with endosulfan sulfate formation and S-mephenytoin N-demethylase activity of CYP2B6 (r^2 = 0.79) while a moderate correlation with testosterone 6-β-hyroxylase activity of CYP3A4 (r^2 = 0.54) was observed. Ticlopidine (5 μM), a potent CYP2B6 inhibitor, and ketoconazole (10 μM), a selective CYP3A4 inhibitor, together inhibited approximately 90% of endosulfan-α metabolism in HLMs. Using six HLM samples, the percent total normalized rate (% TNR) was calculated to estimate the contribution of each CYP in the total metabolism of endosulfan-α. In five of the six HLMs used, the percent inhibition (% I) with ticlopidine and ketoconazole in the same incubation correlated with the combined % TNRs for CYP2B6 and CYP3A4. This study shows that endosulfan-α is metabolized by HLMs to a single metabolite, endosulfan sulfate, and that it has potential use, in combination with inhibitors, as an *in vitro* probe for CYP2B6 and 3A4 catalytic activities.

INTRODUCTION

Endosulfan is an organochlorine pesticide and a contaminant at toxic superfund sites. It is currently applied as a broad spectrum insecticide to a variety of vegetables, fruits, cereal grains, and cotton (USEPA, 2002). Endosulfan is sold under the tradename of Thiodan and as a mixture of two isomers, namely 70% α- and 30% β-endosulfan (ATSDR, 2000). Endosulfan exposure has been shown to increase rodent liver weights and elevate microsomal enzyme levels (Gupta and Gupta, 1977). In mice, endosulfan exposure resulted in increased testosterone metabolism and clearance (Wilson and LeBlanc, 1998). Studies involving children suggest that long term environmental exposure to endosulfan causes delayed male sexual maturation and reduced testosterone levels (Saiyed et al., 2003). The mechanism by which endosulfan may exert these effects may involve its ability to activate the human pregnane X receptor (PXR) and induce the expression levels of cytochrome P450 (CYP or P450) enzymes, thereby increasing metabolic rates.

Prior to beginning an investigation of endosulfan's possible endocrine disrupting effects, we wished to examine its metabolic pathway in humans. Until recently, there has be no published data on human metabolism of endosulfan nor on the possible contributions of CYP isoforms to its metabolism. Based on animal studies, a proposed metabolic pathway for endosulfan was published by the Agency for Toxic Substances and Disease Registry (ATSDR, 2000) and is shown in **Fig.1**. A study using cats reported the immediate presence of endosulfan sulfate in the liver following intravenous administration of endosulfan (Khanna et al., 1979). In rats administered with a single oral dose of ^{14}C-endosulfan, the metabolites sulfate, lactone, ether, and diol were detected in their feces five days later (Dorough et al., 1978). Analyses of human

adipose tissue, placenta, umbilical cord serum, and milk samples demonstrated the presence of parent compound (α and β-endosulfan) and metabolites endosulfan sulfate, diol, lactone, and ether, albeit the sulfate was the predominant degradation product (Cerrillo et al., 2005). The present study determined that endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, in human liver microsomes and its metabolism is primarily mediated by CYP2B6 (at high efficiency) and CYP3A4 (at low efficiency). This is in agreement with results of a recently published study, which also reported the selective metabolism of endosulfan-β by CYP3A4 and CYP3A5 (Lee et al., 2006).

CYP2B6 is recognized to be expressed at only 3 to 5 % of total P450s in human livers (Gervot et al., 1999; Lang et al., 2001) while CYP3A4 is known as the most abundant P450 isoform, expressed at 20-60% of total P450s in human liver (Guengerich, 1995). The respective levels of CYP2B6 and CYP3A4 in human liver microsomes in combination with their strong affinity to endosulfan-α (K_m = 16.2 and 14.4 μM, respectively) and their corresponding clearance rates of endosulfan (CL_{int} = 0.70 and 0.09 μL/min/pmol CYP, respectively) presented a unique opportunity of investigating the potential of endosulfan-α to simultaneously probe for the *in vitro* catalytic activity of both CYP2B6 and 3A4.

MATERIALS and METHODS

Chemicals. Endosulfan-α, the predominant isomer (70 %) in commercial endosulfan, was used in the study of endosulfan metabolism. Endosulfan-α, endosulfan sulfate, endosulfan diol, endosulfan ether, and endosulfan lactone reference materials were purchased from ChemService (West Chester, PA). Stock solutions of endosulfan-α and metabolites were prepared in acetonitrile (ACN) and stored at -20 °C. $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade water, ACN, EDTA, magnesium chloride, Tris, and all other chemicals not specified were purchased from Fisher Scientific (Pittsburgh, PA).

Ticlopidine, a potent mechanism-based chemical inhibitor to CYP2B6 (Richter et al., 2004), and ketoconazole, a selective chemical inhibitor to CYP3A4 (Baldwin et al., 1995) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of ticlopidine were prepared in distilled water and stored at room temperature. Ketoconazole was dissolved in methanol and stock solutions were stored at 4°C.

Human Liver Microsomes (HLMs) and CYP isoforms. Pooled HLMs (20 mg/mL) and 16 selected individual HLMs (20 mg/mL each) were purchased from BD Biosciences. The individual HLMs chosen for this study were representative of the levels of S-mephenytoin N-demethylase activity of CYP2B6 as follows: (Low) HG32, HG95, HH47, HG74, HK37; (Mid) HG43, HG93, HH18, HK25, HH101, HG3; and (High) HH13, HG89, HG64, HG112, HG42. Human recombinant CYP (rCYP) and recombinant flavin monooxygenase (rFMO) isoforms expressed in baculovirus-infected insect cells (supersomes) were also purchased from BD Biosciences.

Metabolism assays. Preliminary studies were performed to determine the times and HLM protein concentrations which produced a linear metabolic rate for 50 μM of endosulfan-α. Endosulfan sulfate formation was linear from 0.05 to 0.25 mg/mL protein and from 5 to 60 min of incubation. The solvent effects of dimethyl sulfoxide (DMSO), acetone, acetonitrile (ACN), methanol, ethanol, and isopropanol at 1% solvent concentration were also tested on endosulfan-α metabolism. There were no differences in the rates of endosulfan sulfate formation among the different solvents, with the exception of isopropanol which slightly inhibited formation of endosulfan sulfate (data not shown).

Based on the results of initial studies, 20 μM endosulfan-α substrate concentration dissolved in ACN, 0.25 mg/mL protein concentration, and 30 min incubation time were used for subsequent metabolism assays, unless otherwise stated. Metabolism assays with HLMs utilized 100 mM potassium phosphate buffer (pH 7.4). Metabolism with rCYPs and rFMOs utilized the following buffers as recommended by BD Biosciences: 100 mM potassium phosphate (pH 7.4) for 1A1, 1A2, 3A4, 3A7, 2D6*1, 3A5, and SF9 insect control; 50 mM potassium phosphate (pH 7.4) for 2B6, 2C8, 2C19, and 2E1; 100 mM tris (pH 7.4) for 2C9*1, 2C18, and 4A11; 50 mM tris (pH 7.4) for 2A6; and 50 mM glycine (pH 9.5) for FMOs 1, 3, and 5. All buffers contained 3.3 mM $MgCl_2$ and 1mM EDTA.

A pre-incubation mixture of endosulfan-α (20 μM), HLMs (0.25 mg/mL) or rCYP isoforms (12.5 pmol), and buffer was prepared in 1.5 mL microcentrifuge tubes. This mixture was pre-incubated for 3 min at 37°C waterbath with minimal agitation. NADPH-regenerating system (final concentration of 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate-dehydrogenase) was added to initiate reaction. The final assay volume was 250 μL. Reactions were carried out for 30 min and terminated with 250 μL cold ACN, followed by pulse-

vortexing. Samples were centrifuged at 16,000 rpm for 5 minutes and supernatants were analyzed in HPLC, as described in the HPLC analysis section below.

Inhibition studies. Protocols for CYP2B6 and CYP3A4 inhibition by ticlopidine and ketoconazole utilized methods previously established by Richter et al, 2004 and Nomeir et al., 2001, respectively. In the case of ticlopidine, a mechanism-based inhibitor of CYP2B6, a 3 min pre-incubation at 37°C of ticlopidine (5 μM) with HLMs (100 μg) or rCYPs (5 pmol) in 50 mM potassium phosphate buffer (with 3.3 mM $MgCl_2$ and 1mM EDTA) in combination with an NADPH regenerating system (final concentration of 0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, and 4 U/mL glucose-6-phosphate dehydrogenase), occurred prior to the addition of endosulfan-α (20 μM). In the case of ketoconazole, endosulfan-α (20 μM) and ketoconazole (10 μM) were pre-incubated along with 100 μg HLMs or 5 pmol rCYP in 50 mM potassium phosphate buffer for 3 min at 37°C prior to the addition of the NADPH regenerating system (final concentration of 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate-dehydrogenase). In both cases, final reaction volumes were 250 μL and reactions were terminated by the addition of 250 μL cold ACN and processed as previously described.

High-performance liquid chromatography (HPLC) analysis. Metabolite formation was analyzed with a Shimadzu HPLC system consisting of an auto-injector (SIL-10AD VP), two pumps (LC-10AT), and a UV detector (SPD-10A VP). Endosulfan-α and metabolites were separated by a Gemini C18 column, 5μm, 100 x 4.6 mm (Phenomenex) and identified with direct injection of reference compounds. The mobile phase for pump A consisted of 99% water and 1% phosphoric acid (pH 2.0) and for pump B, 100% ACN. The flow rate was 1 mL/min. A gradient methodology was used as follows: 0 to 3 minutes (60% ACN), 3 to 16 minutes (60-90% ACN), 16 to 19 minutes (90-60% ACN), and 19 to 20 minutes (60% ACN). The injection

volume was 50 μL and solutes were detected at 213 nm. Under these conditions, the retention times for endosulfan-α and endosulfan sulfate were 12.4 and 8.9 minutes, respectively. Endosulfan-α and endosulfan sulfate peaks were quantified with calibration curves constructed from known concentrations of reference materials. The detection limit for endosulfan sulfate following the US Environmental Protection Agency's method detection limit procedure was 0.04 uM (CFR, 2006).

Data Analyses. Michaelis-Menten and Eadie-Hofstee plots were generated using Sigma Plot Enzyme Kinetics Module (Chicago, IL). Enzyme kinetic parameters K_m and V_{max} were determined using non-linear regression analysis with the Sigma Plot software.

Correlations of endosulfan sulfate formation with each CYP-specific catalytic activity or CYP contents were calculated with simple linear regression using the web-based Statcrunch program (www.statcrunch.com). $p<0.05$ was considered statistically significant.

To estimate the contributions of different CYP isoforms to metabolism of endosulfan-α, percent total normalized rates (% TNR) were calculated using the method described by Rodrigues, 1999. Briefly, metabolite formation rate (pmol/min/pmol rCYP) obtained from rCYP metabolism of the compound of interest is multiplied by the immunoquantified CYP content (pmol nCYP/mg) in native human liver microsomes, yielding the "normalized rate" (NR) expressed in pmol/min/mg microsomes. The NRs for each CYP involved in the metabolism of the compound of interest is summed up as the "total normalized rate" (TNR) (Rodrigues, 1999). The % TNR for each CYP was then calculated according to the following equation.

$$\%\,TNR = \frac{NR}{TNR} \times 100 = \frac{pmol/\min/pmol\,rCYP \times pmol\,nCYP/mg}{\sum(pmol/\min/pmol\,rCYP \times pmol\,nCYP/mg)} \times 100$$

RESULTS

Metabolism of endosulfan-α. Endosulfan-α at 50 μM concentration was metabolized by pooled human liver microsomes (pHLM) to a single metabolite, endosulfan sulfate. **Fig. 2** shows a representative HPLC chromatogram of this metabolism assay. The retention times for endosulfan-α and endosulfan sulfate were 12.23 and 8.73 min, respectively, in a 20 min HPLC run.

Cytochrome P450 screening. Cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) contributions to metabolism of endosulfan-α (20 uM) were investigated using 14 rCYPs and 3 rFMO commercially available human isoforms. Recombinant CYP2B6 predominantly mediated the formation of endosulfan sulfate by 8-fold (at 6.9 nmol/min/nmol CYP) over the next isoform (CYP3A4) with the next highest metabolite formation rate (at 0.8 nmol/min/nmol CYP). CYPs 2C18, 2C19, 2C9*1, and 3A7 also showed metabolic activity, but at negligible levels (**Fig. 3**).

Kinetics of endosulfan-α metabolism. The kinetic parameters K_m and V_{max} were determined by incubating endosulfan-α (0.78-100 μM) with pHLM (0.25 mg/mL), r-CYP2B6 or r-CYP3A4 (12.5 pmol). Calculated apparent K_m, V_{max}, and CL_{int} are shown in **Table 1**.

The respective Michaelis-Menten (M-M) and Eadie-Hofstee plots of endosulfan-α metabolism by pHLM, r-CYP2B6 and r-CYP3A4 are shown in **Fig. 4A-C**. The M-M plot show a hyperbolic curve, indicating saturation of metabolite formation over the substrate concentration range used and suggesting that the data obeyed M-M kinetics. The Eadie-Hofstee plots were linear, indicating either involvement of one enzyme or of more than one enzyme with similar affinity (Ward et al., 2003), and with a slight hook at the bottom end of the curve, suggesting allosteric activation (Faucette et al., 2000).

Correlation of endosulfan sulfate formation with specific CYP contents and selective CYP activities. Endosulfan-α metabolism was conducted in 16 individual HLMs. Results of correlations between selective CYP activities from these 16 individual HLMs and specific CYP contents (of a subgroup of 8 HLMs with immunoquantified CYP contents from BD Biosciences) are shown in **Table 2**. Strong correlations were evident between endosulfan sulfate formation and CYP2B6 and 3A4 contents as determined by immunoquantitation ($r^2 = 0.86$ and 0.81, respectively). Likewise, there was a strong correlation between endosulfan sulfate formation and S-mephenytoin N-demethylase activity of CYP2B6 ($r^2 = 0.79$). A less significant correlation was found with testosterone 6-β-hyroxylase activity of CYP3A4 ($r^2 = 0.54$). No significant correlations were found for the other selective CYP activities and respective contents.

Inhibition of endosulfan-α metabolism by ticlopidine and ketoconazole, selective chemical inhibitors for CYP2B6 and 3A4, respectively. Initially, the optimal concentrations of ticlopidine and ketoconazole needed to obtain maximal inhibition of endosulfan sulfate formation were tested in rCYP2B6 and rCYP3A4. Results of these experiments are shown in **Fig. 5A-B**. It was determined that 5 uM ticlopidine and 10 uM ketoconazole were optimal for subsequent inhibition studies.

Results of inhibition of endosulfan sulfate formation with ticlopidine (5 μM) or/and ketoconazole (10 μM) are shown in **Table 3.** Six individual HLMs were chosen for these studies, based on available immunoquantified CYP contents data supplied by manufacturer. These individual HLMs also represented various ranges of CYP contents (see **Table 4**). Inhibition of endosulfan sulfate formation by ketoconazole among the six individuals varied from 9 to 38%, implicating varying levels of CYP3A4 among these individuals. Similarly, the range of

CYP2B6 involvement varied from 33 to 80%. The results show that inhibition of endosulfan metabolism with ketoconazole and ticlopidine were generally additive in all six HLMs.

Percent Total Normalized Rate (% TNR). % TNR was calculated to verify the percent inhibition (% I) results from this study (**Table 4**). % TNR obtained from rCYPs can be directly related to % I obtained with native HLMs (Rodrigues, 1999).

The % I from the combined incubation with ketoconazole and ticlopidine matched the sum of % TNRs of CYP2B6 and 3A4 in the metabolism of endosulfan-α in five of the six HLMs in this study (see **Table 5**).

DISCUSSION

In the present study, we found endosulfate sulfate as the only metabolite of endosulfan from incubations with HLMs. In mice exposed to a single dose of ^{14}C-endosulfan, endosulfan sulfate concentrations were elevated in the liver, intestine, and visceral fat after 24 hours (Deema et al., 1966). A study in rats administered a single oral dose of ^{14}C-endosulfan showed that the endosulfan metabolites diol, sulfate, lactone, and ether were found in the feces five days later (Dorough et al., 1978). A recent study conducted in Spain where endosulfan is commonly used identified parent endosulfan and metabolites diol, sulfate, lactone and ether in adipose tissues, placenta, cord blood and human milk (Cerrillo et al., 2005). These findings coupled with results of our study suggest that the diol, ether, and lactone metabolites may be the result of metabolic processes beyond those occurring in human liver microsomes.

Our kinetic studies with human liver microsomes as well as with CYP isoforms 2B6 and 3A4 produced monophasic Eadie-Hofstee plots, suggesting that endosulfan-α is metabolized either by one enzyme or by more than one enzyme with similar K_m. A survey of 14 CYP isoforms demonstrated significant metabolism by CYP2B6, followed by 3A4, members of the 2C family and 3A7. Of these isoforms, CYP2B6 and 3A4 are likely to have the greatest impact based upon activity levels and relative abundance. Although CYP2C18 may be similar to CYP3A4 in capacity to metabolize endosulfan, it is poorly expressed in human livers (Goldstein, 2001). Our kinetic studies demonstrated that CYP2B6 and CYP3A4 share similar binding affinities (Km of 16.2 and 14.4 μM, respectively) but vary significantly in maximum velocity. The resulting difference in clearance of endosulfan sulfate demonstrates that CYP2B6 is 8-fold more efficient than CYP3A4 in catalyzing the metabolism of endosulfan-α (see **Table 1**).

Initial inhibition studies utilizing monoclonal antibodies to CYP2B6 and 3A4 were abandoned due to their poor ability to inhibit endosulfan sulfate formation in the recombinant CYP isoforms (less than 30%; data not shown). This suggests that these monoclonal antibodies, although specific in inhibiting the metabolism of some substrates, may not be optimal inhibitors for endosulfan or other substrates. Hence, we used ticlopidine and ketoconazole, selective chemical inhibitors for CYP2B6 and 3A4 respectively, to characterize the contributions of these isoforms to endosulfan-α metabolism. At the concentrations used, these inhibitors did not significantly inhibit the activity of the other isoform examined (**Fig 5**). It is of interest that in the six HLMs examined, the combined use of ketoconazole and ticlopidine resulted in inhibition of endosulfan sulfate formation which was generally similar to the results obtained with each inhibitor alone. For four individuals, the combined inhibition of CYP2B6 and 3A4 yielded values from 85 to 92%, yet two individuals retained significant ability to metabolize endosulfan following inhibition (HK23 and HG93 with 57 and 76% inhibition, respectively). To further explore the possibility that other CYPs were involved in metabolism for these individuals, the total normalized rates of metabolism for the CYP isoforms identified by screening efforts were investigated.

The % I from the combined incubation with ketoconazole and ticlopidine corresponded well with the combined % TNRs of CYP2B6 and 3A4 (**Table 5**) in the metabolism of endosulfan-α in five of the six HLMs in this study. It seemed that with HK23, there was a significantly lower % inhibition of endosulfan-α metabolism by CYP2B6 (as demonstrated by % I with ticlopidine) when compared to the metabolic contribution of CYP2B6 as predicted by % TNR. This decreased inhibition of CYP2B6 activity in HK23 may be due to a CYP2B6 polymorphism. This is supported by a study in which a 26 % decrease was seen in N, N', N''-triethylene-

thiophosphoramide (tTEPA) inactivation of O-deethylation of 7-ethoxy-4-(trifluoromethyl)coumarin (7-EFC) in mutant CYP2B6 compared to wildtype 2B6 (Bumpus et al., 2005). It is now known that CYP2B6 polymorphisms are common in Caucasians and that CYP2B6 is one of the most polymorphic human P450s (Lang et al., 2001).

A number of substrate probes for CYP2B6 have been reported in the literature, including 7-ethoxy-4-trifluoromethylcoumarin (Code et al., 1997), cyclophosphamide and ifosfamide (Huang et al., 2000), S-mephenytoin (Heyn et al., 1996; Ko et al., 1998), bupropion (Faucette et al., 2000; Hesse et al., 2000), and efavirenz (Ward et al., 2003). The known substrate probes for CYP3A4 include testosterone, midazolam, nifedipine, and erythromycin (Yuan et al., 2002). The use of one substrate to simultaneously probe for the *in vitro* catalytic activity of CYP2B6 and CYP3A4 would be very advantageous. Based on the results of our inhibition studies, endosulfan-α appears to be a strong candidate for this role.

In conclusion, endosulfan-α is metabolized to a single metabolite, endosulfan sulfate, by HLMs. This metabolism is primarily mediated by CYP2B6 and CYP3A4. The strategies employed to demonstrate this were: 1) endosulfan-α metabolism by rCYPs, 2) correlation studies of endosulfan sulfate formation and CYP-selective activities or CYP immunoquantified contents in individual HLMs, and 3) inhibition studies using CYP2B6 and CYP3A4 selective chemical inhibitors. In addition, endosulfan-α may be utilized to simultaneously probe for the in-vitro catalytic activities of CYP2B6 and CYP3A4. Finally, endosulfan's endocrine disrupting effects and mechanisms inducing microsomal enzyme activity are currently under investigation.

Acknowledgments. We would like to thank Ed Croom, Amin Usmani, Yan Cao, Leslie Tompkins, and Beth Cooper for their technical assistance.

REFERENCES

ATSDR (2000) Toxicological Profile for Endosulfan, Agency for Toxic Substances and Disease Registry (ATSDR), U.S. Department of Health and Human Services.

Baldwin SJ, Bloomer JC, Smith GJ, Ayrton AD, Clarke SE and Chenery RJ (1995) Ketoconazole and sulphaphenazole as the respective selective inhibitors of P4503A and 2C9. *Xenobiotica* **25:**261-270.

Bumpus NN, Sridar C, Kent UM and Hollenberg PF (2005) The naturally occurring cytochrome P450 (P450) 2B6 K262R mutant of P450 2B6 exhibits alterations in substrate metabolism and inactivation. *Drug Metab Dispos* **33:**795-802.

Cerrillo I, Granada A, Lopez-Espinosa MJ, Olmos B, Jimenez M, Cano A, Olea N and Fatima Olea-Serrano M (2005) Endosulfan and its metabolites in fertile women, placenta, cord blood, and human milk. *Environ Res* **98:**233-239.

CFR (2006) Appendix B to Part 136 - Definition and Procedure for the Determination of the Method Detection Limit - Revision 1.11, Electronic Code of Federal Regulations, Title 40, Feb 2006.

Code EL, Crespi CL, Penman BW, Gonzalez FJ, Chang TK and Waxman DJ (1997) Human cytochrome P4502B6: interindividual hepatic expression, substrate specificity, and role in procarcinogen activation. *Drug Metab Dispos* **25:**985-993.

Deema P, Thompson E and Ware GW (1966) Metabolism Storage and Excretion of C14-Endosulfan in Mouse. *Journal of Economic Entomology* **59:**546-&.

Dorough HW, Huhtanen K, Marshall TC and Bryant HE (1978) Fate of Endosulfan in Rats and Toxicological Considerations of Apolar Metabolites. *Pesticide Biochemistry and Physiology* **8:**241-252.

Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laethem RM and Lindley CM (2000) Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab Dispos* **28:**1222-1230.

Gervot L, Rochat B, Gautier JC, Bohnenstengel F, Kroemer H, de Berardinis V, Martin H, Beaune P and de Waziers I (1999) Human CYP2B6: expression, inducibility and catalytic activities. *Pharmacogenetics* **9:**295-306.

Goldstein JA (2001) Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* **52:**349-355.

Gupta PK and Gupta RC (1977) Effect of endosulfan pretreatment on organ weights and on pentobarbital hypnosis in rats. *Toxicology* **7:**283-288.

Hesse LM, Venkatakrishnan K, Court MH, von Moltke LL, Duan SX, Shader RI and Greenblatt DJ (2000) CYP2B6 mediates the in vitro hydroxylation of bupropion: potential drug interactions with other antidepressants. *Drug Metab Dispos* **28:**1176-1183.

Heyn H, White RB and Stevens JC (1996) Catalytic role of cytochrome P4502B6 in the N-demethylation of S-mephenytoin. *Drug Metab Dispos* **24:**948-954.

Huang Z, Roy P and Waxman DJ (2000) Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide. *Biochem Pharmacol* **59:**961-972.

Khanna RN, Misra D, Anand M and Sharma HK (1979) Distribution of endosulfan in cat brain. *Bull Environ Contam Toxicol* **22:**72-79.

Ko JW, Desta Z and Flockhart DA (1998) Human N-demethylation of (S)-mephenytoin by cytochrome P450s 2C9 and 2B6. *Drug Metab Dispos* **26:**775-778.

Lang T, Klein K, Fischer J, Nussler AK, Neuhaus P, Hofmann U, Eichelbaum M, Schwab M and Zanger UM (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver. *Pharmacogenetics* **11:**399-415.

Lee HK, Moon JK, Chang CH, Choi H, Park HW, Park BS, Lee HS, Hwang EC, Lee YD, Liu KH and Kim JH (2006) Stereoselective Metabolism of Endosulfan by Human Liver Microsomes and Human Cytochrome P450 Isoforms. *Drug Metab Dispos.*

Nomeir AA, Ruegg C, Shoemaker M, Favreau LV, Palamanda JR, Silber P and Lin CC (2001) Inhibition of CYP3A4 in a rapid microtiter plate assay using recombinant enzyme and in human liver microsomes using conventional substrates. *Drug Metab Dispos* **29:**748-753.

Richter T, Murdter TE, Heinkele G, Pleiss J, Tatzel S, Schwab M, Eichelbaum M and Zanger UM (2004) Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. *J Pharmacol Exp Ther* **308:**189-197.

Rodrigues AD (1999) Integrated cytochrome P450 reaction phenotyping - Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochemical Pharmacology* **57:**465-480.

Saiyed H, Dewan A, Bhatnagar V, Shenoy U, Shenoy R, Rajmohan H, Patel K, Kashyap R, Kulkarni P, Rajan B and Lakkad B (2003) Effect of endosulfan on male reproductive development. *Environ Health Perspect* **111:**1958-1962.

USEPA (2002) Reregistration Eligibility Decision (R.E.D) for Endosulfan, U.S. Environmental Protection Agency (USEPA).

Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA and Desta Z (2003) The cytochrome P4502B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: Implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of

CYP2B6 catalytic activity. *Journal of Pharmacology and Experimental Therapeutics* **306:**287-300.

Wilson VS and LeBlanc GA (1998) Endosulfan elevates testosterone biotransformation and clearance in CD-1 mice. *Toxicol Appl Pharmacol* **148:**158-168.

Yuan R, Madani S, Wei XX, Reynolds K and Huang SM (2002) Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* **30:**1311-1319.

Footnotes:

This work was supported by NIOSH grant OH 07551-ECU. R.C. was a recipient of the Air Force Institute of Technology scholarship. Results were presented at the 45th annual meeting of the Society of Toxicology in San Diego, CA, 2006.

Send reprint requests to: Randy L. Rose, PhD, Department of Environmental and Molecular Toxicology, Box 7633, North Carolina State University, Raleigh, NC 27695.

Figure legends:

Fig. 1. The proposed metabolic pathway for endosulfan based on animal studies, as published by ATSDR, 2000, was modified to show that human CYP2B6 and CYP3A4 primarily catalyzes the metabolism of endosulfan-α to endosulfan sulfate, the only metabolite detected in the present study.

Fig. 2. A representative HPLC chromatogram of endosulfan-α metabolism to endosulfan sulfate, the lone metabolite detected in incubations with human liver microsomes. The three peaks towards the end of the chromatogram were determined to be contributions from human liver microsomes.

Fig. 3. Rates of endosulfan sulfate formation from endosulfan-α (20 μM) by 14 recombinant cytochrome P450s (rCYPs) and 3 recombinant flavin monoxygenase (r-FMO) isoforms. Data shown are the means of two independent determinations.

Fig. 4. Velocity of endosulfan sulfate formation versus endosulfan-α concentration in human liver microsomes (A), recombinant CYP2B6 (B), and recombinant CYP3A4 (C). Each point represents the mean of three independent measures.

Fig. 5. Inhibition of endosulfan sulfate formation in rCYP2B6 and rCYP3A4 by (A) ketoconazole (0-10 μM) and (B) ticlopidine (0-10 μM). Each point represents the mean of two independent measures.

TABLE 1

Kinetic parameters of endosulfan-α metabolism in pooled human liver microsomes (pHLM), recombinant CYP2B6 and 3A4

HLMs or CYP	K_m (μM)	V_{max}	CI_{int}
pHLM	9.8	178.5[a]	18.20[c]
CYP2B6	16.2	11.4[b]	0.70[d]
CYP3A4	14.4	1.3	0.09

[a] *Vmax* expressed in pmol/min/mg protein for pHLM and in pmol/min/pmol CYP for CYP2B6 and 3A4

[b] Vmax expressed in pmol/min/pmol CYP for CYP2B6 and 3A4

[c] Intrinsic clearance (Vmax/Km) expressed in μL/min/mg protein for pHLM

[d] Intrinsic clearance (Vmax/Km) expressed in μL/min/pmol CYP for CYP2B6 and 3A4

TABLE 2

Correlation of rates of endosulfan sulfate formation and CYP-selective activities or CYP-specific contents

Rate of endosulfan sulfate formation from 50 μM endosulfan-α was determined in 16 individual human liver microsomes (HLMs). Determinations were done in two independent experiments. The rates of CYP-selective activities and CYP-specific contents for these 16 HLMs were supplied by the manufacturer. Statistical analyses were done using simple non-linear regression in the web-based StatCrunch program. $p < 0.05$ was considered statistically significant.

	Rate of Endosulfan sulfate formation (pmol/min/mg)	
Rates of CYP-selective activities (pmol/min/mg) or CYP content (pmol/mg)	r^2	p
s-Mephenytoin N-demethylase activity of 2B6	0.79	<0.0001
Testosterone 6-B-hydroxylase activity of 3A4	0.55	0.001
Diclofenac-4-hydroxylase activity of 2C9	0.04	0.460
s-Mephenytoin 4-hyroxylase activity of 2C19	0.01	0.743
2B6 content	0.86	0.0008
3A4 content	0.81	0.002
2C9 content	0.42	0.167
2C19 content	0.01	0.571

Table 3

Inhibition of E sulfate formation in HLMs by Ketoconazole and Ticlopinid

Inhibitors ketoconazole (10 uM) and ticlopidine (5 uM) were used alone and combined for metabolism of endosulfan-α in six individual human liver microsomes (HLMs). Data are means of two independent measurements.

	% Inhibition of Endosulfan sulfate formation					
Inhibitor	**HG3**	**HG112**	**HG42**	**HG43**	**HG93**	**HK23**
10 uM KTZ	23.8 ± 0.6	20.5[a]	8.6 ± 1.5	34.9 ± 4.7	37.6 ± 8.8	36.0 ± 0.2
5 uM TCL	67.8 ± 2.6	64.4 ± 0.5	79.2 ± 3.0	57.0 ± 0.4	38.6 ± 3.3	33.0 ± 4.8
5 uM TCL + 10 uM KTZ	92.3 ± 0.4	88.0 ± 1.3	91.5 ± 2.4	85.2 ± 0.6	75.6 ± 6.2	57.0 ± 0.6

[a]No replicate for this measurement due to insufficient HLM HG112 sample.

TABLE 4

Comparison between % Total Normalized Rates (% TNR) and % Inhibition (% I) in three human liver microsomes (HLMs)

% TNR was calculated according to Rodrigues et al, 1999. % I for CYP2B6 was determined with the use of ticlopidine (5 μM) and for CYP3A4 with ketoconazole (10 μM).

HLMs	Recombinant CYP (rCYP)	E sulfate formation rate[a] in rCYP	CYP content[b] in native HLMs	Normalized Rate	% TNR	% I
HG42	2B6	9.42	53	499.37	64.8	79.2
	2C9	0.34	80	24.12	3.5	ND[c]
	2C19	0.32	6	1.89	0.2	ND
	3A4	0.78	310	242.73	31.5	8.6
HG112	2B6	9.42	47	442.83	58.8	64.4
	2C9	0.34	87	29.49	3.9	ND
	2C19	0.32	72	22.68	3.0	ND
	3A4	0.78	330	258.39	34.3	20.5
HG3	2B6	9.42	18	169.60	65.0	67.8
	2C9	0.34	42	14.24	5..4	ND
	2C19	0.32	9	2.84	1.1	ND
	3A4	0.78	95	74.38	28.5	23.8
HK23	2B6	9.42	7	65.95	42.1	33.0
	2C9	0.34	56	18.98	12.1	ND
	2C19	0.32	17	5.36	3.4	ND
	3A4	0.78	85	66.56	42.4	36.0
HG43	2B6	9.42	4	37.69	26.3	57.0
	2C9	0.34	51	17.29	12.1	ND
	2C19	0.32	47	14.81	10.3	ND
	3A4	0.78	94	73.60	51.3	34.9
HG93	2B6	9.42	18	169.60	69.0	38.6
	2C9	0.34	51	17.29	7.3	ND
	2C19	0.32	49	15.44	6.7	ND
	3A4	0.78	52	40.72	17.0	37.6

[a] Rates in pmol/min/pmol rCYP
[b] Immunoquantified CYP contents in pmol/min/mg protein
[c] Not determined.

TABLE 5

Sum of CYP2B6 and 3A4 % TNRs vs. % I with Ketoconazole and Ticlopidine

Comparison between the sum of % Total Normalized Rates (%TNRs) of CYP2B6 and CYP3A4 in the metabolism of Endosulfan-α and % Inhibition (% I) with ketoconazole and ticlopidine in the same incubation. With the exception of HK23, the other 5 HLMs had matching % TNR and % I.

HLM	% TNR	% I
HG3	94	92
HG112	93	88
HG42	96	92
HK23	84	57
HG43	78	85
HG93	86	76

Fig. 1.

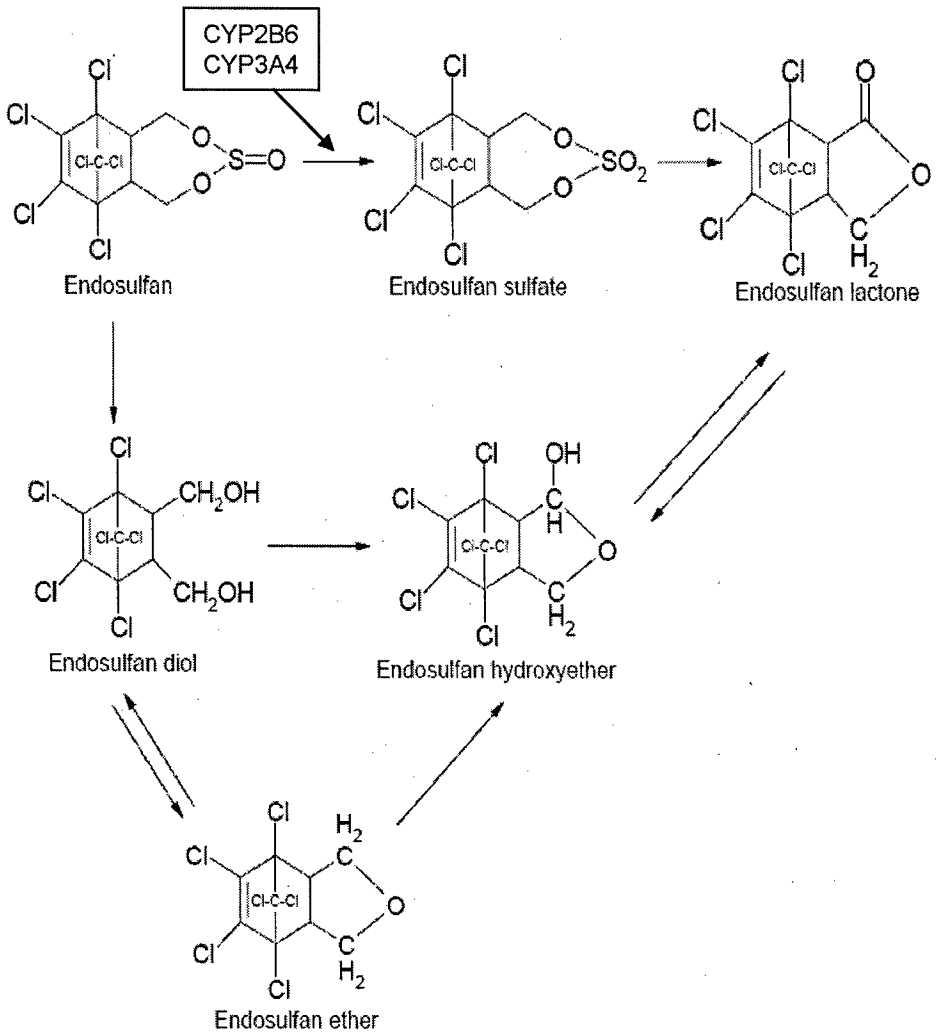

CYP2B6
CYP3A4
Endosulfan
Endosulfan sulfate
Endosulfan lactone
Endosulfan diol
Endosulfan hydroxyether
Endosulfan ether

Fig. 2.

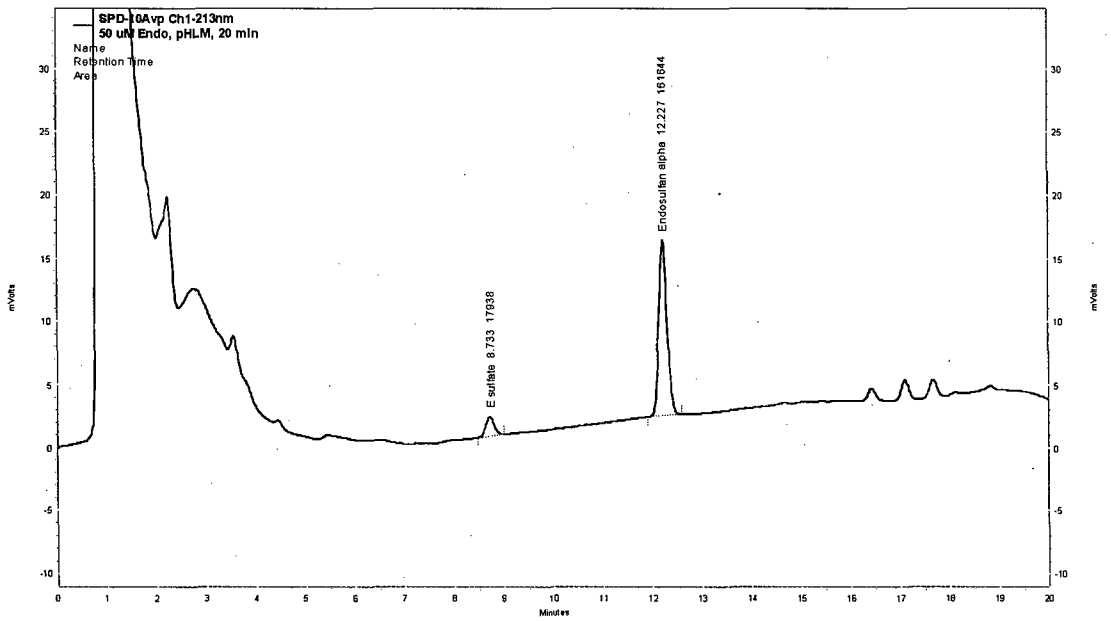

SPD-10Avp Ch1-213nm
50 uM Endo, pHLM, 20 min
Name
Retention Time
Area
Endosulfan alpha 12.227 161844
E sulfate 8.733 17938
mVolts
Minutes

Fig. 3.

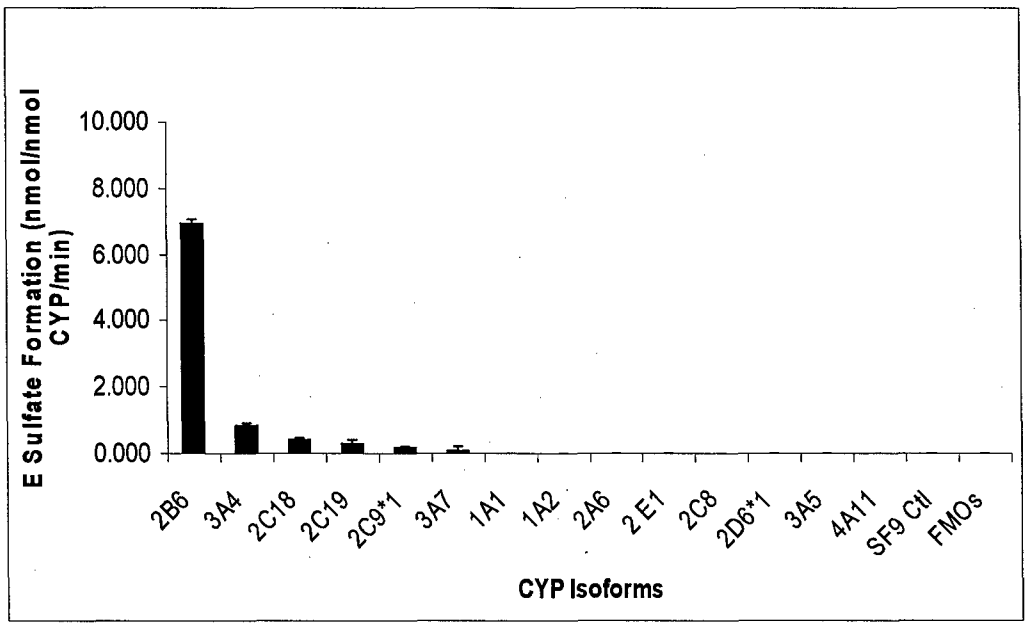
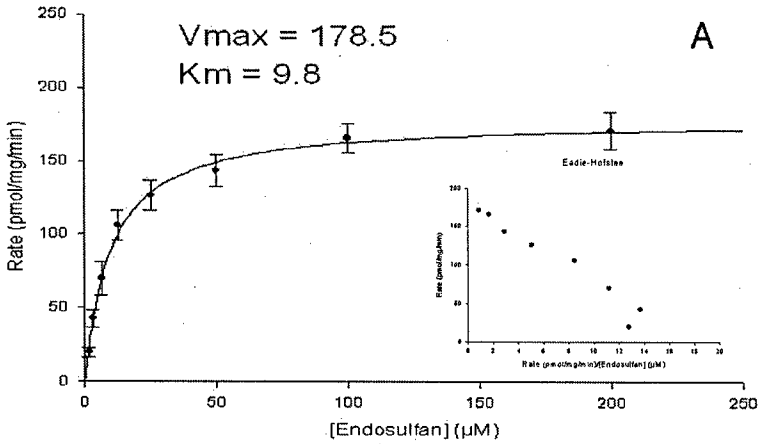
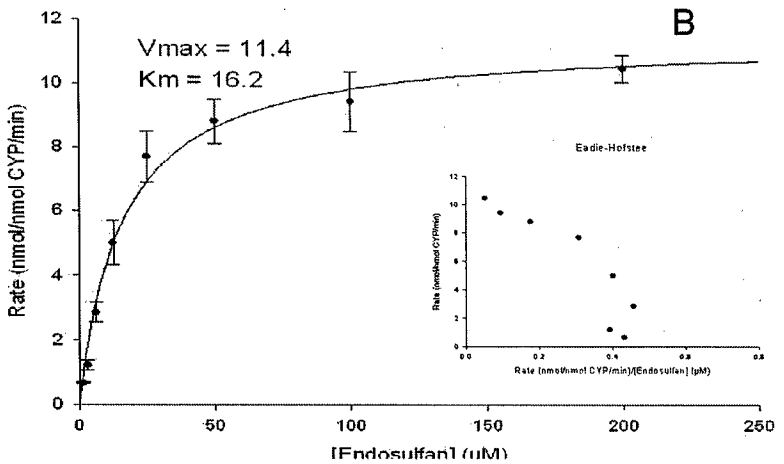

E Sulfate Formation (nmol/nmol CYP/min)
10.000
8.000
6.000
4.000
2.000
0.000
2B6
3A4
2C18
2C19
2C9*1
3A7
1A1
1A2
2A6
2 E1
2C8
2D6*1
3A5
4A11
SF9 Ctl
FMOs
CYP Isoforms

Fig. 4


Vmax = 178.5
Km = 9.8
A
Rate (pmol/mg/min)
[Endosulfan] (µM)
Eadie-Hofstee


Vmax = 11.4
Km = 16.2
B
Rate (nmol/nmol CYP/min)
[Endosulfan] (µM)
Eadie-Hofstee

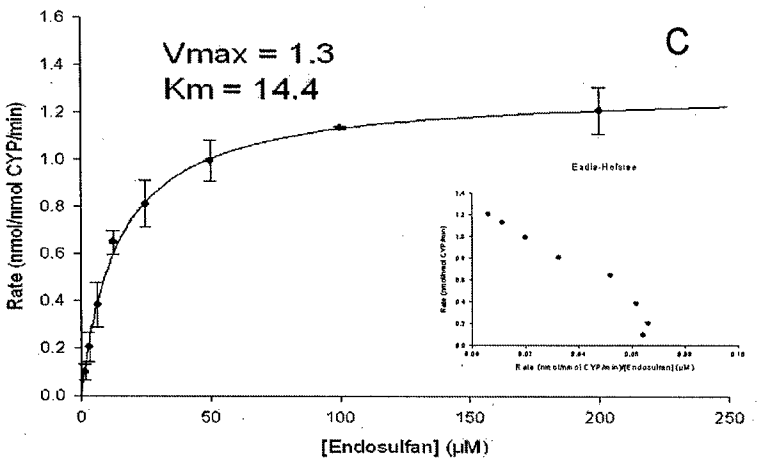
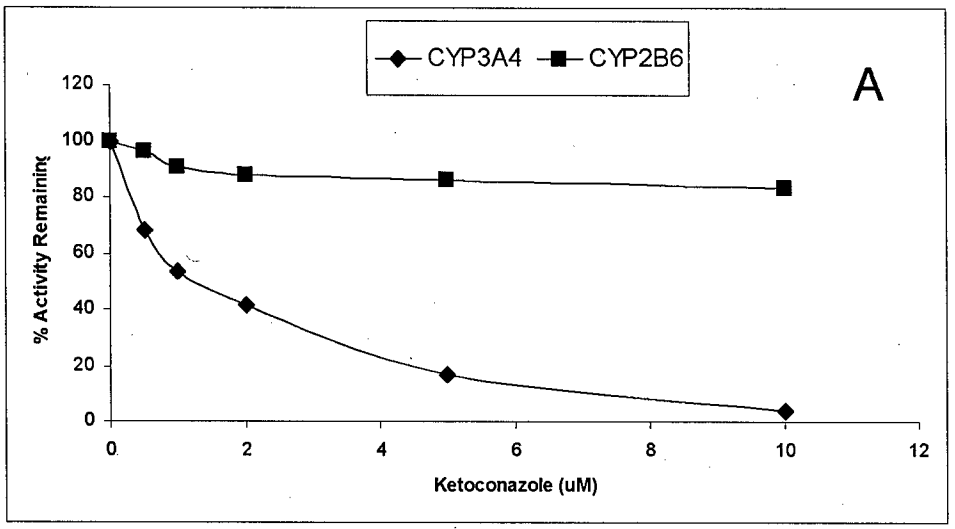
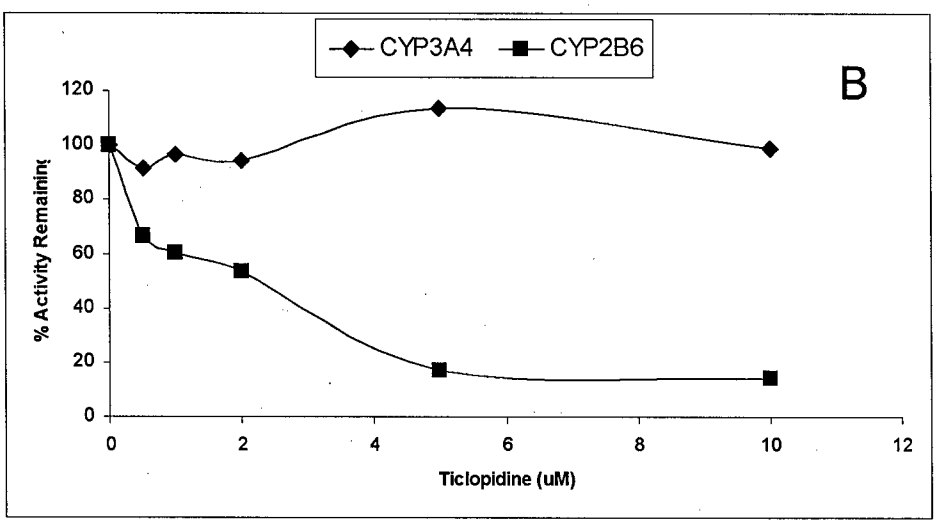

Vmax = 1.3
Km = 14.4
C
Rate (nmol/nmol CYP/min)
[Endosulfan] (µM)
Eadie-Hofstee

Fig. 5


CYP3A4
CYP2B6
A
% Activity Remainin
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Ketoconazole (uM)


CYP3A4
CYP2B6
B
% Activity Remainin
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Ticlopidine (uM)

CHAPTER 2

ENDOSULFAN-ALPHA INDUCES CYP2B6 AND CYP3A4 BY ACTIVATING THE PREGNANE X RECEPTOR BUT NOT THE CONSTITUTIVE ANDROSTANE RECEPTOR

ABSTRACT

Endosulfan is an organochlorine pesticide and a contaminant at toxic superfund sites. It is postulated that its endocrine disrupting properties may be due to its ability to induce testosterone metabolism by inducing cytochrome P450 enzymes. The present study demonstrated that endosulfan-α induces CYP3A4 and CYP2B6 by activating the pregnane X receptor (PXR), but not the constitutive androstane receptor (CAR). These interactions were explored by transient transfection assays in the HepG2 cell line using CYP3A4-luciferase, CYP2B6-luciferase, human PXR (hPXR), and mouse CAR (mCAR) plasmids. Endosulfan-α (10 μM) treatments resulted in 11-fold induction of CYP3A4 and 16-fold induction of CYP2B6 promoter activities over untreated controls in the presence of hPXR. The metabolite endosulfan sulfate (10 μM) also induced CYP3A4 and CYP2B6 promoter activities by 6-fold and 12-fold, respectively. In the presence of mCAR and androstenol, a mCAR repressor, endosulfan-α induced CYP2B6 promoter activity by only 3-fold over control. Western blots utilizing S9 samples from primary human hepatocytes showed that endosulfan-α induces CYP2B6 translation in a dose-dependent manner (0.1 to 50 μM) and CYP3A4 translation, but only at 50 μM dose. Testosterone (TST) metabolism assays using the same S9 samples revealed an increase of TST metabolites, albeit only at 50 μM endosulfan-α treatment. This study has determined that endosulfan-α activates the hPXR, but

not mCAR, and induces the CYP2B6 and CYP3A4 promoter activity and protein expression, that endosulfan-α may induce hepatic toxicity via apoptotic process, and that it may affect endocrine homeostasis by inducing P450s and consequently increasing testosterone metabolism.

INTRODUCTION

Endosulfan is an organochlorine pesticide that is commonly applied to vegetables (such as squash and pumpkins), fruits (such as cantaloupe, honeydew and strawberries) and cotton (USEPA, 2002). It is sold under the tradename of Thiodan®, which is a mixture of 70% α- and 30% β-endosulfan. Endosulfan is also found as a contaminant in soil and groundwater, transported through spray drift and runoff water (ATSDR, 2000). It is absorbed by both humans and animals percutaneously and through ingestion and inhalation (Kalender et al., 2004). In laboratory animals, endosulfan produces toxicity to the liver, kidney, nervous system, and reproductive organs (ATSDR, 2000).

Low dose endosulfan exposures modify endogenous antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione S transferase (GST) which leads to development of oxidative stress in different tissues (Bebe and Panemangalore, 2003). Endosulfan also acts as an estrogen agonist, triggering the formation of increased numbers of alveolar buds and telomerase reverse transcriptase (TERT) mRNA expression in mouse mammary glands (Je et al., 2005). It induces free radical metabolism, cardiotoxicity, and shows toxic interactions with the biochemistry and histology of liver and kidney in rats (Choudhary et al., 2003; Kalender et al., 2004). Endosulfan induces testicular

damage (abnormal spermatozoa, decreasing sperm count and sperm motility) in developing rats (Rao et al., 2005), and testicular toxicity in adult rats (Jaiswal et al., 2005).

Endosulfan has also been suggested to be an endocrine disruptor. In mice, endosulfan treatments increased testosterone metabolism and clearance (Wilson and LeBlanc, 1998). In male rats exposed to endosulfan, a dose related decrease in testosterone was seen (Singh and Pandey, 1990). Another study in rats demonstrated that endosulfan induced P450s in hepatic and extra-hepatic tissues (Siddiqui et al., 1987). Studies in children suggest that chronic exposure to endosulfan caused decrease in testosterone and delayed male sexual maturity (Saiyed et al., 2003).

Endocrine disrupting chemicals have been shown to activate the PXR and/or CAR, which are nuclear receptors that appear to protect the integrity of the endocrine system by serving as sensors of xenobiotics and, in turn, inducing detoxification enzymes such as CYP2B and CYP3A (Kretschmer and Baldwin, 2005). Endosulfan has been reported to induce the CYP3A4 promoter activity by activating hPXR in studies utilizing either transient transfection or a stable HepG2 cell line expressing hPXR and CYP3A4 luciferase reporter gene (Coumoul et al., 2002; Lemaire et al., 2004). No report was found in the literature on endosulfan's ability to activate CAR.

The pregnane X receptor (PXR; also known as the steroid and xenobiotic receptor, SXR) and the constitutive androstane receptor (CAR) are members of the orphan nuclear receptor family. They play important roles in the body's detoxification mechanism by serving as sensors of toxic substances and subsequently inducing the production of metabolizing enzymes (Blumberg et al., 1998; Kliewer et al., 1998; Kretschmer and Baldwin, 2005). PXR and CAR are activated by xenobiotics (foreign compounds) and once activated, they form a

heterodimer with the 9-cis retinoic acid receptor (RXR) then bind to response elements of xenobiotic metabolizing enzymes, including several phase I cytochrome P450 enzymes (CYP), phase II enzymes, and transporters (Kliewer et al., 2002; Kretschmer and Baldwin, 2005). Recently, studies in human hepatocytes found that CYP2B6 and CYP3A4 are non-selectively induced by activated PXR, but CYP2B6 is preferentially induced (over CYP3A4) by activated CAR (Faucette et al., 2006).

CYP enzymes are members of a superfamily of hemeproteins that play an important role in the human metabolism of drugs and xenobiotics and in the synthesis of steroid hormones (Estabrook, 2003; Nelson, 2003). CYP3A4 is the most abundant P450 in the human liver and small intestine and plays a major role in the metabolism of approximately 50% of drugs used today (Guengerich, 1999) as well as in the metabolism of endogenous substances such as steroid hormones (Usmani et al., 2003). Studies using human liver microsomes reported that CYP3A4 is the major CYP isoform responsible for the metabolism of testosterone through hydroxylation at the 6-β position (Waxman et al., 1988; Usmani et al., 2003). In a previous study in our laboratory, it was demonstrated that endosulfan-α is metabolized primarily by CYP2B6 and secondarily by CYP3A4 (Casabar et al., 2005). This was confirmed by a recently published study, which also reported the selective metabolism of endosulfan-β by CYP3A4 and CYP3A5 (Lee et al., 2006).

The purpose of the present study was to investigate if endosulfan-α increases the metabolism of testosterone through the activation of PXR and/or CAR and subsequent induction of cytochrome P450 (CYP) enzymes. The methods used for this investigation were: 1) toxicity assays to determine the dose-response of endosulfan-α that induces toxic effects to the hepatic cells, 2) transfection assays in HepG2 cells and luciferase assays to

demonstrate the ability of endosulfan-α to activate the human pregnane X receptor (hPXR) and/or the mouse constitutive androstane receptor (mCAR) and regulate the CYP2B6 and CYP3A4 promoter activities, 3) western blotting to detect inducibility of CYP2B6 and CYP3A4 in primary human hepatocytes treated with endosulfan-α, and 4) testosterone hydroxylation assays using S9 preparations from primary human hepatocytes treated with endosulfan-α to assess functionality of induced CYP2B6 and CYP3A4 enzymes.

MATERIALS and METHODS:

Chemicals, reagents, DNA primers and cell media. Endosulfan-α , endosulfan-β, technical-grade endosulfan (60:40 mixture of endosulfan-α and β isomers), and endosulfan sulfate were purchased from ChemService (West Chester, PA) and stock solutions were dissolved in ethanol or acetonitrile (ACN). 5α-androst-16-en-3α-ol (Androstenol) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) were obtained from Sigma-Aldrich (St. Louis, MO). Androstenol was dissolved in ethanol and TCPOBOP was dissolved in DMSO, with dilutions prepared in ethanol. Rifampicin (Rif), dexamethasone, phenobarbital (PB), and all other chemicals, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO). The ToxiLightTM assay kit was purchased from Cambrex Corporation (East Rutherford, New Jersey). Caspase-GloTM 3/7 assay kit was purchased from Promega Corporation (Madison, WI). Actinomycin D and Z-DEVD-FMK are the products of Alexis Biochemicals supplied by AXXORA, LLC (San Diego, CA).

Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). High fidelity PCR system was purchased from Roche Diagnostics. DNA ligation and gel purification systems were purchased from Promega. *E. coli* competent cells (GC5) were

purchased from GeneChoice. TA cloning kit and restriction endonucleases were purchased from Invitrogen. Plasmid purification kit was purchased from Qiagen.

Cell culture media were purchased from Cellgro, unless stated otherwise. TransIT transfection reagent was purchased from Mirus Corp (Madison, WI). Luciferase reporter and β-galactactosidase assay system with reporter lysis buffer were purchased from Promega (Madison, WI). Polyclonal anti-human CYP2B6 from rabbit and monoclonal anti-human CYP3A4 from mice were purchased from BD Biosciences (Woburn, MA).

Biological materials: The human liver carcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC). Primary human hepatocytes were purchased from ADMET Technologies (Research Triangle Park, NC).

Plasmids Constructs: The β-galactactosidase and the firefly luciferase reporter plasmids pGL3 and pGL4 basic vectors were purchased from Promega. The plasmids CYP3A4-Luciferase and pSG5-hPXR were provided by Dr. Jean Marc Pascussi, and the pSG5-mCAR was provided by Dr. Andrew Wallace. The CYP3A4-luciferase construct contains the proximal (-263 to +11) ER6 motif and distal xenobiotic response element (XREM) containing a DR3 motif (**Fig 1A**). The CYP2B6 PBREM-XREM-luciferase reporter plasmid was cloned as described below.

CYP2B6 promoter cloning and pGL4-PBREM-XREM-luciferase construct. The location and sequences of the proximal phenobarbital responsive enhancer module (PBREM) and distal xenobiotic responsive enhancer module (XREM) regions in the CYP2B6 promoter were previously characterized by Wang *et al*, 2003. The pGL4 luciferase reporter vector was utilized to construct a reporter plasmid which contained the proximal PBREM (with two DR4 motifs) and the distal XREM (with a DR4 motif) of the CYP2B6 promoter (**Fig 1B**).

The distal XREM region was PCR-amplified from human genomic DNA with the forward primer 5'-CGGGGTACCCTTTCTCCATCCACAAAATCG-3' (with Kpn I restriction site) and the reverse primer 5'-CGCTCGAGGATGCTGATTCAGGGAATCCA-3' (with Xho I restriction site). A 410 bp product which contained the responsive elements was generated and subcloned into a pCR 2.1 TA vector. The TA plasmid with XREM insert was then transformed into competent *E. coli* cells, cloned, purified, and sent out for sequencing. After confirmation with sequencing results and with diagnostic digests using Kpn I and Xho I, stock *E. coli* cells containing plasmids with XREM insert were stored in -80°C.

The proximal CYP2B6 PBREM was PCR-amplified from human genomic DNA with the primers 5'-GAAGATCTCTGCAATGAGCACCCAATCTT-3' (forward primer, with Bgl II restriction site) and 5'-CCCAAGCTTCTGCACCCTGCTGCAGCCTCC-3' (reverse primer, with Hind III restriction site). A 1.8 kb product was generated and subcloned into a pCR 2.1 TA vector. The TA plasmid with PBREM insert was then transformed into competent *E. coli* cells, cloned, purified, and sent out for sequencing. After confirmation with diagnostic digests using Bgl II and HindIII and with sequencing results, stock *E. coli* cells containing plasmids with the PBREM insert were stored in -80°C.

PBREM and XREM inserts were cut, purified, and ligated into the pGL4 vector. First, pGL4 with PBREM insert was cloned in competent GC5 *E. coli* cells. Then, XREM was ligated into the pGL4 containing the PBREM insert. Finally, the pGL4-XREM-PBREM plasmid was cloned in competent *E. coli* cells and purified.

Endosulfan toxicity studies. Cell viability was assessed using cells harvested as a cell suspension in isotonic culture medium. The trypan blue exclusion assay was used to semi-quantitatively determine the viability of cells in all treatment groups and controls, using a

hemocytometer. Specifically, 100 µl of 0.4% Trypan blue in PBS (pH 7.4) was added into 900 µl of cell suspension. 10 µl of this mixture was placed on the hemocytometer for counting under the microscope. Greater than 100 cells per field were examined and the data was expressed as percent viable cells (Hausser Scientific, Horsham, PA).

Endosulfan toxicity to HepG2 and primary human hepatocytes were assessed using the ToxiLight and Caspase-3/7 assay systems. ToxiLightTM is a non-destructive luciferase based bioluminescence cytotoxicity assay used to measure toxicity in mammalian cells and cell lines in culture. The kit quantitatively measures the release of adenylate kinase into the culture medium. The emitted light intensity expressed as RLU (relative luminescence unit) value is linearly related to the adenylate kinase activity. The assay was performed according to the manufacturer's protocol (Cambrex Bio Science Rockland, Inc., Rockland, ME). Caspase-GloTM-3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and 7 activities by using a luminometer. Luminescence produced by luciferase as RLU (relative luminescence unit) value is proportional to the amount of caspase-3/-7 activity present in the sample. The assay was performed according to the manufacturer's protocol (Tech. Bull. No.323, Promega Corporation, Madison, WI).

Induction of CYP2B6 and 3A4 promoter activity via hPXR. HepG2 cells were utilized for transient transfection with the CYP2B6 pGL4-XREM-PBREM-Luc or CYP3A4 pGL3-XREM-Luc reporter vectors and human PXR expression vector.

HepG2 cells were maintained in EMEM with 10% fetal bovine serum, 1% glutamine, 1% penstrep, 1% sodium pyruvate, and 1% non-essential amino acids. Prior to transfection, HepG2 (3×10^5 cells per well) were plated into six-well plates. Twenty-four hours later, the media was removed, cells were rinsed with Optimem, then 1 mL Optimem was added into

each well before addition of the transfection mixture. Following the manufacturer's recommendation, a transfection mixture of Transit reagent (Mirus Corp) and Optimem media were incubated together for 5 min. Then expression plasmids pSG5 or pSG5-hPXR, β-galactactosidase (0.1 μg per well), and reporter plasmids CYP2B6 pGL4-XREM-PBREM-Luc or CYP3A4-Luc (1.0 μg per well) were added into the transfection mixture and incubated together for 20 min at RT. Finally, the transfection mixture was added into each well. Four to six hours later, the media was changed to Complete serum-free media (2 mL per well). On day 3, the cells were treated with endosulfan-α, β, technical-grade endosulfan or endosulfan sulfate (0.1% treatment volume or 2 μL of 10 mM endosulfan per well). On day 4, media was removed and cells were rinsed with 1 mL PBS. Cell lysates were prepared according to Promega's luciferase assay system protocol. Briefly, 400 μL of reporter lysis buffer (1x) were added into each well and a single freeze-thaw cycle was performed. Lysates were transferred into 1.5 mL eppendorf tubes, vortexed for 10-15 sec, then centrifuged at 12,000 × *g* for 2 min (at 4°C). Supernatants were transferred into new tubes and stored at -80 °C for future luciferase assays.

The luciferase assay (Promega) was utilized to measure induction of CYP2B6 and CYP3A4 promoter activity. In 1.5 mL eppendorf tubes, 5 μL of cell lysates were added into 40 μL of luciferase reagent and luciferase activity was read immediately in a Turner luminometer.

The β-galactosidase assay system (Promega) was used to measure amount of β-galactosidase plasmid transfection. In 1.5 mL eppendorf tubes, 75 μL of cell lysates were added into into 75 μL β-galactosidase assay buffer (2X). Reactions were carried out for 30 min or until the appearance of a yellow coloration at 37°C waterbath. Reactions were

terminated with the addition of 250 μL of sodium carbonate (1 M). β-galactosidase activity was read in a spectrophotometer at 420 nm. The β-galactosidase activity readings were then used to normalize the luciferase activity.

Induction of CYP2B6 promoter activity via mCAR. The protocol for this experiment is the same as the above, with the following exceptions. The expression plasmid pSG5-mCAR was transfected into HepG2 cells, instead of the pSG5-hPXR, 24 hrs before treatment of cells. The cells were treated with 4 μM androstenol alone, or both androstenol and 0.25 μM TCPOBOP, or both androstenol and 10 μM endosulfan-α for 24 hrs. All treatments had 0.1% solvent concentration per well.

Induction of CYP2B6 and 3A4 in endosulfan-treated primary human hepatocytes. Primary human hepatocytes purchased from ADMET were plated (1.5×10^6 / well) in 6-well culture plates coated with Collagen Type I and overlayed with Matrigel. The hepatocytes were equilibrated in a humidified incubator at 5% CO_2 / 95% air at 37°C for 72 hrs and cultured in Williams' medium E (2 mL per well), which was replaced every 24 hrs prior to treatment. The media was supplemented with penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL), dexamethasone (10^{-7} M), insulin (10^{-7} M), and 10% FBS.

Hepatocytes were treated with either rifampicin (10 μM) or phenobarbital (100 μM) or increasing concentrations of endosulfan-α (0.1, 1, 5, 10, and 50 μM) every 24 hrs over a 2 day period. Control cells were treated with ACN at 0.1% solvent concentration.

Cells were harvested from 2 wells of a 6-well plate using a cell scraper and then pooled in eppendorf tubes for protein extraction. Cells in eppendorf tubes were then centrifuged at 5000 *g* for 3 min and the supernatant was discarded. The cells were then suspended in 75 μL chilled cytochrome P450 storage buffer (0.1 M potassium phosphate buffer with 0.1 mM

EDTA at pH 7.5) and sonicated twice for 30 sec. S9 microsomal protein was extracted by centrifuging the sample at 9000 *g* for 15 minutes. The protein concentrations of S9 samples were measured using the Bio-Rad protein assay. Briefly, 5 μL of S9 sample or 10 μL protein standard (0, 0.25, 0.5, 1, 2 mg/mL prepared from 10 mg/mL BSA) were added to a 600 μL of dye:water mixture (1:4). The reaction was incubated for 5 min at RT and absorbance was read in a spectrophotometer at 590 nm. Protein concentrations of S9 samples were calculated from a curve generated from the absorbance readings of BSA standard samples.

Western blot. The levels of CYP2B6 and CYP3A4 expressed in primary hepatocytes treated with different doses of endosulfan-α were detected by western blotting, using S9 fractions. 8% Tris Glycine pre-cast 10-well gels (Invitrogen) were used for electrophoresis of protein samples at a constant voltage of 125 V for 1.5 to 2 hrs. Proteins from the gel were then transferred onto a nitrocellulose membrane by electroblotting at a constant voltage of 105 V for 90 min. The blots were blocked with a 10% membrane blocking agent (Amersham Biosciences) in TBST (10 mmol/L Tris HCL pH 7.5, 140 mmol/L sodium chloride, and 0.1% Tween 20) for 90 min with gentle rocking. Then the blots were rinsed 3 times (5 min each) in TBST with 1% nonfat dry milk. The membranes were incubated with the primary antibody anti-human CYP2B6 rabbit monoclonal antibody or anti-human CYP3A4 mouse monoclonal antibody in TBST with 1% nonfat dry milk overnight at 4°C with gentle rocking. The membranes were rinsed in TBST with 1% nonfat dry milk and incubated with a secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse, respectively) for 90 min at RT with gentle rocking. After washing membranes two times (5 min each) in TBST with 1% nonfat dry milk, then once (5 min) with TBST, each blot was

incubated with 3 mL of 1:1 ECL reagents (Amersham Biosciences) for 2 min then exposed to autoradiography films for detection of specific protein bands.

Testosterone metabolism assay. The *in vitro* metabolism of testosterone (TST) and HPLC detection of metabolites used a modification of a protocol previously described by Usmani *et al*, 2003. A pre-incubation mixture of S9 samples (100 μg) or CYP2B6 (10 pmoles) with an NADPH-regenerating system (final concentration of 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, and 2 U/mL glucose-6-phosphate-dehydrogenase), and 50 mM potassium phosphate buffer (pH 7.4) was prepared in 1.5 mL microcentrifuge tubes and pre-incubated for 5 min at 37°C with minimal agitation. TST (200 μM) was then added into the mixture. The final assay volume was 250 μL. Reactions were carried out for 30 min and terminated with 250 μL cold methanol, followed by pulse-vortexing. Samples were centrifuged at 16,000 rpm for 5 minutes and supernatants were analyzed by HPLC.

TST metabolites were detected with a Shimadzu HPLC system consisting of an auto-injector (SIL-10AD VP), two pumps (LC-10AT), and a UV detector (SPD-10A VP). TST and metabolites were separated by a Prodigy column (3 μ, 150 x 4.6 mm, ODS, 100 Ao; Phenomenex, Rancho Palos Verdes, CA) and identified with direct injection of reference compounds. Mobile phase for pump A was 1% tetrahydrofuan, 99% water; for pump B 100% methanol. The flow rate was 0.5 mL/min. The injection volume was 50 μL and solutes were detected at 247 nm. A gradient methodology was used as follows: 0 to 14 min (60-85% B), 14 to 17 min (85-60% B), 17 to 20 min (60% B). TST and metabolites were quantified with standard curves based on peak area.

RESULTS:

CYP2B6 promoter construct. The 410 bp XREM and 1.8 kb PBREM were inserted into the pGL4 basic vector at the restriction sites Kpn I/Xho I and Bgl II/Hind III, respectively, then ligating the inserts in the plasmid with DNA ligase. **Fig 1B** shows a map of the CYP2B6 XREM/PBREM Luciferase construct.

Effect of endosulfan-α on adenylate kinase and caspase-3/7 activities and cell viability in HepG2 cells and human hepatocytes. Cytotoxicity has been shown to effect PXR reporter gene assays in studies of various PXR ligands (Vignati et al., 2004). Assessment of endosulfan-α cytotoxicity was done to ensure that the PXR activation was not underestimated due to altered cell viability. Adenylate kinase, an enzyme released from damaged cells, was monitored following treatment with increasing doses of endosulfan-α in cultured HepG2 cells using the Toxilight assay kit (Cambrex Bio Sciences). Endosulfan-α induction of adenylate kinase activity gradually increased with dose, peaking at 50–100 μM with a level of activity of ~2-2.5 fold above control levels at 72 h (**Fig 2A**). Parallel experiments were conducted using human hepatocytes from two different individuals and data were summarized. Adenylate kinase activity increased dose-dependently up to 50 μM, however dropped at 100 μM at 48 and 72 h (**Fig 2B**).

Caspase-3/7 activity is one of the important markers of the cellular apoptotic process. In order to verify whether endosulfan-α mediated cell death was triggered through the known apoptotic pathway, cultured HepG2 cells were exposed to increasing concentrations of endosulfan-α (1 to 100 μM) for 24, 48 and 72 h and caspase-3/7 activity was determined. Time- and dose-dependent induction of caspase-3/7 activity was noted from 1 to 12.5 μM endosulfan-α, while slowly decreasing from 25 - 100 μM endosulfan-α. The maximum induction was ~4-fold above solvent treated control at 72 h (**Fig 2C**). To verify the data

obtained from HepG2 cells, fresh human hepatocytes were exposed similarly to increasing concentrations of endosulfan-α (1 to 100 μM). Cells were also exposed to known caspase-3/7 inducer Actinomycin D and Z-DEVD-FMK, a known caspase 3/7 specific inhibitior. Data indicated that endosulfan at 50 and 100 μM significantly induced caspase-3/7 activity ~4-fold at 24, 48 and 72 h, however induction of caspase-3/7 activity leveled off at 72 h (**Fig 2D**). Actinomycin D induced caspase-3/7 activity 8 to 25-fold above control (data not shown), whereas Z-DEVD-FMK completely abrogated the endosulfan-induced caspase-3/7 activity (**Fig 2D**).

Endosulfan-α Induces CYP2B6 and CYP3A4 promoter activity in HepG2 Cells via hPXR. To determine if endosulfan is able to activate the human pregnane X receptor (hPXR) and induce CYP2B6 and CYP3A4 promoter activity, reporter assays were performed. HepG2 cells were transiently transfected with pSG5-hPXR and CYP2B6-Luc or CYP3A4-Luc. Transfected cells were treated with the known hPXR agonist Rifampicin (Rif), which is the prototypical inducer of CYP3A4 and has been shown to induce CYP2B6 (Xie et al., 2000; Goodwin et al., 2001; Wang et al., 2003; Lemaire et al., 2004).

Rif (10 μM), in the presence of hPXR, induced CYP2B6 and CYP3A4 promoter activities by 10-fold and 17-fold, respectively, over control (**Fig. 3**). In comparison, endosulfan-α at 10 μM induced CYP2B6 and CYP3A4 promoter activities by 16-fold and 11-fold, respectively, over control. A minimum induction of the CYP2B6 and CYP3A4 promoter activities were seen with 1 μM endosulfan-α and none were seen at 0.1 and 0.01 μM concentrations. Interestingly, the metabolite endosulfan sulfate (10 μM) also induced CYP2B6 and CYP3A4 promoter activities by 12-fold and 6 fold, respectively, over control.

In the absence of hPXR, rifampicin, endosulfan-α and its metabolite endosulfan sulfate did not induce CYP2B6 and CYP3A4 promoter activity.

Technical-grade Endosulfan and Endosulfan-β Induce the CYP3A4 promoter at the same level as Endosulfan-α. In light of the recent report by Lee *at al*, 2003 that endosulfan-β is metabolized primarily by CYP3A4 and CYP3A5, we wanted to investigate if endosulfan-β and technical-grade endosulfan induce the CYP3A4 promoter at a higher level than endosulfan-α, which would then suggest that perhaps an elevated testosterone metabolism that would be caused by endosulfan may be due to an elevated CYP3A4 levels induced by endosulfan-β, moreso than by endosulfan-α. However, our results showed that technical-grade endosulfan and endosulfan-β induction of the CYP3A4 promoter does not differ significantly from induction levels seen with endosulfan-α (**Fig. 4**).

Induction of CYP2B6 and CYP3A4 protein levels in endosulfan-α-treated human hepatocytes. Having shown that endosulfan-α treatment of HepG2 cells transiently transfected with hPXR induced the CYP2B6 and CYP3A4 promoter activity, we wished to determine if endosulfan-α had a similar role in hepatocytes, which are cells that endogenously express PXR. Freshly isolated human hepatocytes were obtained from two individuals to assess the ability of endosulfan-α to induce the protein levels of CYP2B6 and CYP3A4. Endosulfan-α induced CYP2B6 protein expression in primary human hepatocytes in a dose-dependent manner. Endosulfan-α at 10 μM induced CYP2B6 better than 10 μM rifampicin (Rif) or 100 μM phenobarbital (PB) (**Fig 5A**). The actin lane shows equal loading of protein samples. **Fig 5B** shows that CYP3A4 is induced at a significant level only at 50 μM endosulfan-α (no significant inductions were seen at 0.1, 1, 5, and 10 μM concentrations).

Endosulfan-α weakly induces CYP2B6 promoter activity via mCAR. Faucette *et al*, 2006, reported that CYP2B6 and CYP3A4 are non-selectively induced by PXR, but CYP2B6 is preferentially induced (over CYP3A4) by CAR. Because our western blots showed us a preferential induction of CYP2B6 over CYP3A4 at lower endosulfan-α concentrations, we thought that perhaps endosulfan-α had a preferential binding and activation of CAR over PXR. Hence, we investigated if endosulfan-α induction of CYP2B6 is CAR-mediated.

CAR-mediated transactivation is constitutive in HepG2 cells, and the compound androstenol represses the constitutively-active CAR (Kawamoto et al., 1999). TCPOBOP, a potent PB-type inducer, can be used as a positive control in inducing CYP2B6 in androstenol-treated HepG2 (Sueyoshi et al., 1999). In the present study, HepG2 cells were transiently transfected with mCAR expression plasmids and CYP2B6-luc plasmids. The cells were treated with androstenol (4 μM) alone, both androstenol (4 μM) and TCPOBOP (0.25 μM), or both adrostenol (4 μM) and endosulfan-α (10 μM). Our results showed that CYP2B6 promoter activity was induced by 14-fold and 3-fold by TCPOBOP and endosulfan-α, respectively, over androstenol-treated HepG2 cells (**Fig. 6**), indicating that endosulfan-α is a weak activator of CAR.

Testosterone metabolism with S9 samples of endosulfan-α treated primary human hepatocytes. The utility of conducting testosterone assays to monitor CYP3A4 inducibility in human hepatocyte cultures has been demonstrated previously (Fayer et al., 2001). In our studies, two individual human hepatocytes were cultured in the presence of endosulfan-α for 72 hours prior to the determination of testosterone metabolic activity. The retention times of reference testosterone (TST) and metabolites we observed in our HPLC method were as follows: TST (16.6 min), 2-β-hydroxy TST (12.6 min), 11-β-hydroxy TST (11.4 min), 16-β-

hydroxy TST (11.0 min), 6-β-hydroxy TST (8.2 min), and 15-β-hydroxy TST (7.2 min). It has been reported that 6-β-hydroxy TST makes up about 86% of all testosterone metabolites formed by CYP enzymes, and that CYP3A4 mediates 87% of 6-β-hydroxylation of TST (Usmani et al., 2003). In the present study, TST metabolism was measured primarily through the formation of 6-β-hydroxy TST.

With the first individual hepatocytes, no significant levels of TST metabolites were detected at lower doses of endosulfan-α treatments (0.1-10 μM). However, at 50 μM endosulfan-α, a significant increase in TST hydroxylation was detected (**Fig. 7**). With the second individual hepatocytes, no increase in TST hydroxylation were seen with any of the endosulfan-α doses (0.1-50 μM). In both individual hepatocytes, Rif (10 μM) and PB (100 μM), known inducers of CYP3A4 and CYP2B6, respectively, both demonstrated increased levels of TST hydroxylation over control sample.

DISCUSSION:

Endosulfan induces oxidative damage and cytotoxicity in human HepG2 and HeLa cell lines, with IC50 values of 49 μM and 86 μM, respectively (Sohn et al., 2004). Endosulfan also induces testicular toxicity and damage testicular tissue by the process of necrosis (Jaiswal et al., 2005). A recent report indicated endosulfan as a potential carcinogen in humans, inducing the production of reactive oxygen species (ROS) which in turn induce ERK activation, probably by stabilizing its phosphorylation in human HaCaT cells (Ledirac et al., 2005). Oxidative stress caused by ROS is associated with the swelling of the mitochondria which facilitates the cellular loss of ATP.

In the present study, endosulfan-α significantly induced the production of adenylate kinase at higher time points (a marker of ATP loss from cells) and at higher doses (indicative of cytotoxic effects of endosulfan in hepatic cells), in both HepG2 and primary human hepatocytes. Endosulfan-α also significantly induced caspase-3/7 activity in HepG2 and human hepatic cells. However, there was little difference in the inducing potential between hepatoma and primary hepatic cells. Such induction was abrogated with the Caspase-3/7 specific inhibitor, Z-DEVD-FMK, suggesting that endosulfan-α-mediated hepatocyte toxicity occurs via apoptosis.

We have demonstrated that endosulfan-α induced the CYP2B6 and CYP3A4 promoter activities by activating PXR, but only weakly activating CAR. Our findings on the induction of the CYP3A4 promoter via hPXR are in agreement with studies reported by Coumoul *et al*, 2002 and Lemaire *et al*, 2004. Previous studies in our laboratory demonstrated that endosulfan-α is primarily metabolized by CYP2B6, with a minor contribution by CYP3A4 (Casabar et al, 2005). This observation led to our investigation of the potential of endosulfan-α to induce CYP2B6, in addition to CYP3A4. We found that endosulfan-α induced CYP2B6 promoter activity at a higher level than it did with CYP3A4. These results demonstrated that endosulfan-α regulates its own metabolism by inducing CYP2B6 and CYP3A4, which are the main enzymes responsible for its metabolism.

We then explored the potential for endosulfan-α to increase protein expression of CYP2B6 and CYP3A4. Treating primary human hepatocytes with endosulfan-α, we found that endosulfan-α induced CYP2B6 and CYP3A4 protein expression. This is in agreement with the findings of Lemaire et al, 2004. Albeit, we found that endosulfan-α induced CYP3A4 at a significant level only at 50 μM concentration, but not at the lower

concentrations of 0.1, 1, 5, and 10 μM. With CYP2B6, our results show a dramatic dose dependent induction. Even at 0.1 μM dose, endosulfan-α significantly induced CYP2B6 protein expression compared to control hepatocytes.

We further carried out our study to demonstrate the functionality of the CYP2B6 and CYP3A4 proteins induced by endosulfan-α. To study the ability of endosulfan-α to affect substrate metabolism, we incubated testosterone with S9 microsomal samples prepared from endosulfan-α treated (0.1 to 50 μM) primary human hepatocytes. Usmani *et al.*, 2003 reported that the formation of 6-β-hydroxy testosterone metabolite is primarily mediated by CYP3A4. Another study reported that 16-β hydroxylation of testosterone is catalyzed by CYP2B6 and that it may be used as a marker of CYP2B6 catalytic activity (Hanna et al., 2000). So we looked at the levels of 6-β and 16-β hydroxy testosterone metabolites in our HPLC assay to detect induction of CYP3A4 and CYP2B6, respectively, and determine their functionality in catalyzing metabolism of testosterone. Rif (10 μM), a prototypical CYP3A4 inducer, showed an increase in the levels of 6-β hydroxy TST. However, PB (100 μM), a known inducer of CYP2B6 did not demonstrate any increase in 16-β hydroxy TST, although it showed an elevation of 6-β hydroxy TST metabolites. With endosulfan-α treated hepatocyte samples, no TST metabolites were increased at lower doses (0.1-10 μM endosulfan-α). Only at 50 μM endosulfan-α treatment did we observe a significant increase in testosterone hydroxylation, albeit only the 6-β hydroxy TST metabolites was significantly elevated.

The above results suggest that endosulfan-α, at biologically relevant concentrations, may not increase testosterone metabolism in humans. However, our *in vitro* model may not be sufficient to demonstrate actual biological rates of testosterone degradation in humans. In

rats exposed to phenobarbital, a specific increase in the production of 16-β-hydroxy testosterone was seen as a result of induced CYP2B1 and CYP2B2 (Waxman, 1988). In mice exposed to endosulfan, the 16-β, 6-α, and 16α- (but not 6-β) hydroxy testosterone metabolites were elevated in the urine, and this study suggested that endosulfan may have selectively induced P450 activities most indicative of the phenobarbital (PB)-type response (Wilson and LeBlanc, 1998). Another study in mice exposed to the compound mirex reported increases in CYP2B10 protein and in the formation of 16-β hydroxy testosterone metabolite in incubations with mouse liver microsomes, although the study could not directly attribute the increased 16-β hydroxy testosterone to CYP2B10 since the compound mirex also induced other CYP isoforms (Dai et al., 2001). In the present study, we found that endosulfan-α activated hPXR, which binds to PB-type response elements of the CYP2B6 gene and subsequently induced the transcription of the gene. However, endosulfan-α only weakly activated mCAR. This finding is supported by studies of Dr. William S. Baldwin (Univ. of Texas at El Paso) who suggested in his seminar at North Carolina State University that endosulfan weakly activated CYP2B10 in a mouse model with humanized CAR (unpublished report). These findings and ours suggest that endosulfan may increase the biotransformation of testosterone by inducing CYP2B6 protein levels, in addition to induction of CYP3A4, via PXR.

In summary, we present in this paper evidence that endosulfan-α activates the hPXR (but only weakly activates the mCAR) subsequently inducing transcription and translation of CYP3A4 and CYP2B6, and that it regulates its own metabolism possibly causing metabolic interactions with endogenous and exogenous chemicals. We also observed that endosulfan-α may induce hepatic toxicity via apoptotic process. Our findings suggest that endosulfan may

affect endocrine homeostasis by inducing CYP2B6 and CYP3A4 isoforms and consequently increasing testosterone metabolism by hydroxylation.

REFERENCES

ATSDR (2000) Toxicological Profile for Endosulfan, Agency for Toxic Substances and Disease Registry (ATSDR), U.S. Department of Health and Human Services.

Bebe FN and Panemangalore M (2003) Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissues of rats. *J Environ Sci Health B* **38:**349-363.

Blumberg B, Sabbagh W, Jr., Juguilon H, Bolado J, Jr., van Meter CM, Ong ES and Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* **12:**3195-3205.

Casabar R, Wallace A and Rose R (2005) Endosulfan Induces Cytochrome P450-3A4 and 2B6 through the Steroid and Xenobiotic Receptor (Poster Abstract # 244). in: *International Society for the Study of Xenobiotics. Drug Metabolism Reviews. Abstracts from 13th ISSX meeting, October 23-27, 2005.*, pp 141, Maui, HI.

Choudhary N, Sharma M, Verma P and Joshi SC (2003) Hepato and nephrotoxicity in rat exposed to endosulfan. *J Environ Biol* **24:**305-308.

Coumoul X, Diry M and Barouki R (2002) PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. *Biochem Pharmacol* **64:**1513-1519.

Dai D, Cao Y, Falls G, Levi PE, Hodgson E and Rose RL (2001) Modulation of mouse P450 isoforms CYP1A2, CYP2B10, CYP2E1, and CYP3A by the environmental chemicals mirex, 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene, vinclozolin, and flutamide. *Pesticide Biochemistry and Physiology* **70:**127-141.

Estabrook RW (2003) A passion for P450s (rememberances of the early history of research on cytochrome P450). *Drug Metab Dispos* **31:**1461-1473.

Faucette SR, Sueyoshi T, Smith CM, Negishi M, Lecluyse EL and Wang H (2006) Differential Regulation Of Hepatic Cyp2b6 And Cyp3a4 Genes By Constitutive Androstane Receptor But Not Pregnane X Receptor. *J Pharmacol Exp Ther.*

Fayer JL, Petullo DM, Ring BJ, Wrighton SA and Ruterbories KJ (2001) A novel testosterone 6 beta-hydroxylase activity assay for the study of CYP3A-mediated metabolism, inhibition, and induction in vitro. *J Pharmacol Toxicol Methods* **46:**117-123.

Goodwin B, Moore LB, Stoltz CM, McKee DD and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* **60:**427-431.

Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39:**1-17.

Hanna IH, Reed JR, Guengerich FP and Hollenberg PF (2000) Expression of human cytochrome P450 2B6 in Escherichia coli: characterization of catalytic activity and expression levels in human liver. *Arch Biochem Biophys* **376:**206-216.

Jaiswal A, Parihar VK, Sudheer Kumar M, Manjula SD, Krishnanand BR, Shanbhag R and Unnikrishnan MK (2005) 5-Aminosalicylic acid reverses endosulfan-induced testicular toxicity in male rats. *Mutat Res* **585:**50-59.

Je KH, Kim KN, Nam KW, Cho MH and Mar W (2005) TERT mRNA expression is up-regulated in MCF-7 cells and a mouse mammary organ culture (MMOC) system by endosulfan treatment. *Arch Pharm Res* **28:**351-357.

Kalender S, Kalender Y, Ogutcu A, Uzunhisarcikli M, Durak D and Acikgoz F (2004) Endosulfan-induced cardiotoxicity and free radical metabolism in rats: the protective effect of vitamin E. *Toxicology* **202:**227-235.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* **19:**6318-6322.

Kliewer SA, Goodwin B and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev* **23:**687-702.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92:**73-82.

Kretschmer XC and Baldwin WS (2005) CAR and PXR: xenosensors of endocrine disrupters? *Chem Biol Interact* **155:**111-128.

Ledirac N, Antherieu S, d'Uby AD, Caron JC and Rahmani R (2005) Effects of organochlorine insecticides on MAP kinase pathways in human HaCaT keratinocytes: key role of reactive oxygen species. *Toxicol Sci* **86:**444-452.

Lee HK, Moon JK, Chang CH, Choi H, Park HW, Park BS, Lee HS, Hwang EC, Lee YD, Liu KH and Kim JH (2006) Stereoselective Metabolism of Endosulfan by Human Liver Microsomes and Human Cytochrome P450 Isoforms. *Drug Metab Dispos.*

Lemaire G, de Sousa G and Rahmani R (2004) A PXR reporter gene assay in a stable cell culture system: CYP3A4 and CYP2B6 induction by pesticides. *Biochem Pharmacol* **68:**2347-2358.

Nelson D (2003) Cytochrome P450 Homepage (http://drnelson.utmem.edu/CytochromeP450.html).

Rao M, Narayana K, Benjamin S and Bairy KL (2005) L-ascorbic acid ameliorates postnatal endosulfan induced testicular damage in rats. *Indian J Physiol Pharmacol* **49:**331-336.

Saiyed H, Dewan A, Bhatnagar V, Shenoy U, Shenoy R, Rajmohan H, Patel K, Kashyap R, Kulkarni P, Rajan B and Lakkad B (2003) Effect of endosulfan on male reproductive development. *Environ Health Perspect* **111:**1958-1962.

Siddiqui MKJ, Anjum F and Qadri SSH (1987) Some Metabolic Changes Induced by Endosulfan in Hepatic and Extra Hepatic Tissues of Rat. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes* **22:**553-564.

Singh SK and Pandey RS (1990) Effect of Subchronic Endosulfan Exposures on Plasma Gonadotropins, Testosterone, Testicular Testosterone and Enzymes of Androgen Biosynthesis in Rat. *Indian Journal of Experimental Biology* **28:**953-956.

Sohn HY, Kwon CS, Kwon GS, Lee JB and Kim E (2004) Induction of oxidative stress by endosulfan and protective effect of lipid-soluble antioxidants against endosulfan-induced oxidative damage. *Toxicol Lett* **151:**357-365.

Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* **274:**6043-6046.

USEPA (2002) Reregistration Eligibility Decision (R.E.D) for Endosulfan, U.S. Environmental Protection Agency (USEPA).

Usmani KA, Rose RL and Hodgson E (2003) Inhibition and activation of the human liver microsomal and human cytochrome P450 3A4 metabolism of testosterone by deployment-related chemicals. *Drug Metabolism and Disposition* **31:**384-391.

Vignati LA, Bogni A, Grossi P and Monshouwer M (2004) A human and mouse pregnane X receptor reporter gene assay in combination with cytotoxicity measurements as a tool to evaluate species-specific CYP3A induction. *Toxicology* **199:**23-33.

Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M and LeCluyse EL (2003) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* **278:**14146-14152.

Waxman DJ (1988) Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37:**71-84.

Waxman DJ, Attisano C, Guengerich FP and Lapenson DP (1988) Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6 beta-hydroxylase cytochrome P-450 enzyme. *Arch Biochem Biophys* **263:**424-436.

Wilson VS and LeBlanc GA (1998) Endosulfan elevates testosterone biotransformation and clearance in CD-1 mice. *Toxicol Appl Pharmacol* **148:**158-168.

Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS and Evans RM (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev* **14:**3014-3023.

Figure Legends

Fig. 1. Map of (A) CYP3A4-luciferase and (B) CYP2B6-luciferase constructs.

Fig. 2. Endosulfan toxicity in human hepatocytes. Dose and time dependent effect of endosulfan on Adenylate kinase activity in **(A)** human hepatoma (HepG2) and **(B)** human hepatic cells, and on Caspase-3/7 activity in **(C)** human hepatoma HepG2 and **(D)** human hepatic cells. Each RLU value bar represents the mean of 6 from two experiments or 4-well determinations from two individuals and the error bars are the standard error mean in six samples and two individuals, respectively. Data were duplicated in separate experiment.

Fig 3. Endosulfan-α induction of **(A)** CYP3A4 and **(B)** CYP2B6 promoter activity via the human PXR. Data shown are means of three independent measures.

Fig. 4. Endosulfan-β and endosulfan technical-grade induction of the CYP3A4 promoter via the hPXR. Data are means of three independent measurements.

Fig. 5. Western blot showing endosulfan induction of CYP3A4 and CYP2B6 protein expression in primary human hepatocytes. Protein samples used were S9 preparations from primary human hepatocytes.

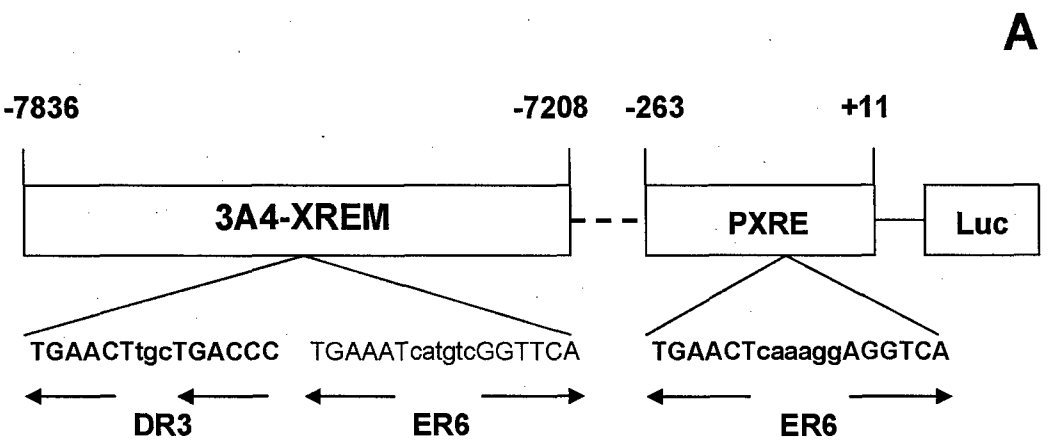
Fig. 6. Endosulfan-α induction of the CYP2B6 promoter via mCAR. Data shown are means of three independent measures.

Fig.7. Testosterone metabolites detected with endosulfan-treated hepatocyte S9 samples.

Fig. 8. A model of PXR and CAR activation by endosulfan-α. Activated CAR translocates to the nucleus, albeit endosulfan-α only weakly activates CAR, which results in weak induction of CYP2B6 (dashed arrows and smaller letters for CAR, relative to PXR, signifies weak activation by endosulfan). Endosulfan-α diffuses to the nucleus and activates PXR. Activated PXR heterodimerizes with RXR and activates the response

elements of both CYP2B6 and CYP3A4. Activated CAR heterodimerizes with RXR and weakly activates the response elements of CYP2B6. Subsequent gene induction occurs, resulting in possible drug-drug interaction or endocrine disruption or excretion of metabolites.

Fig 1.


A
-7836
-7208
-263
+11
3A4-XREM
PXRE
Luc
TGAACTtgcTGACCC
TGAAATcatgtcGGTTCA
TGAACTcaaaggAGGTCA
DR3
ER6
ER6

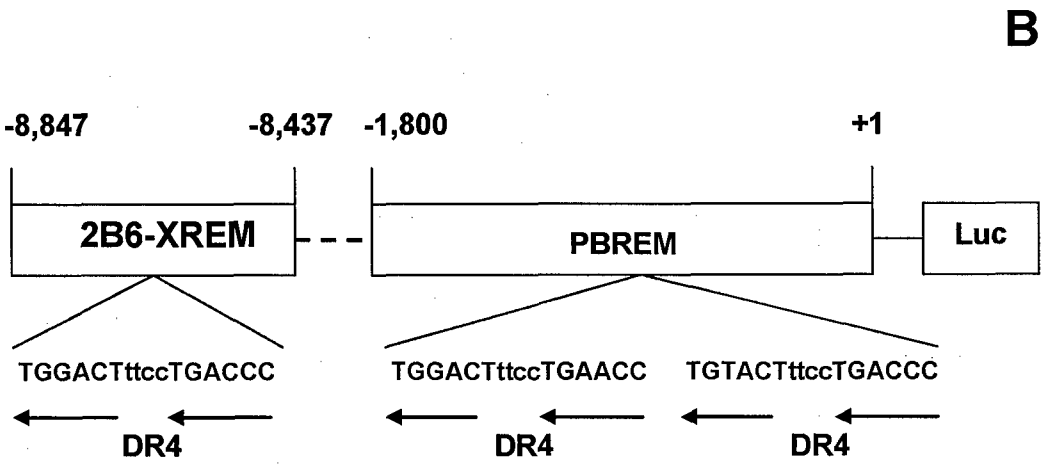

B
-8,847
-8,437
-1,800
+1
2B6-XREM
PBREM
Luc
TGGACTttccTGACCC
TGGACTttccTGAACC
TGTACTttccTGACCC
DR4
DR4
DR4

Fig 2

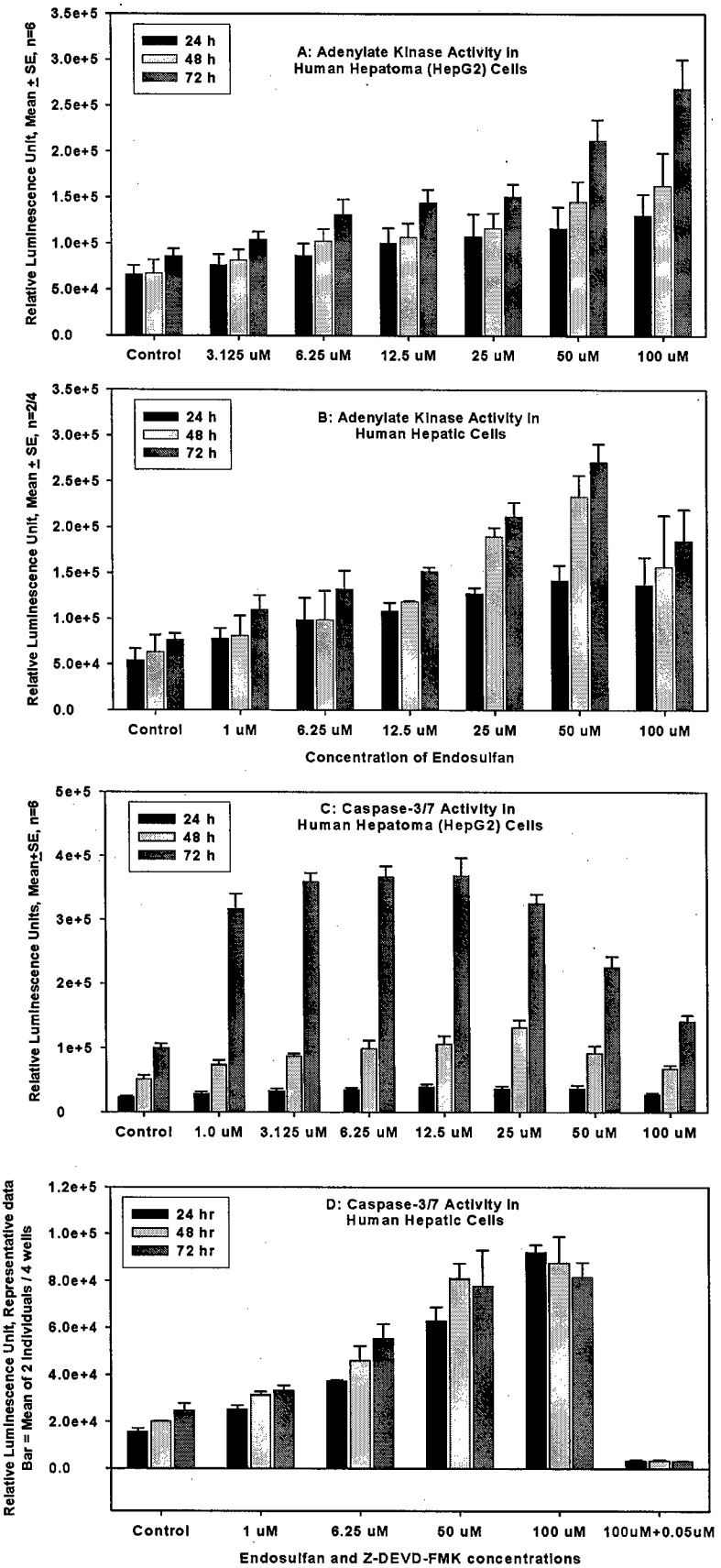
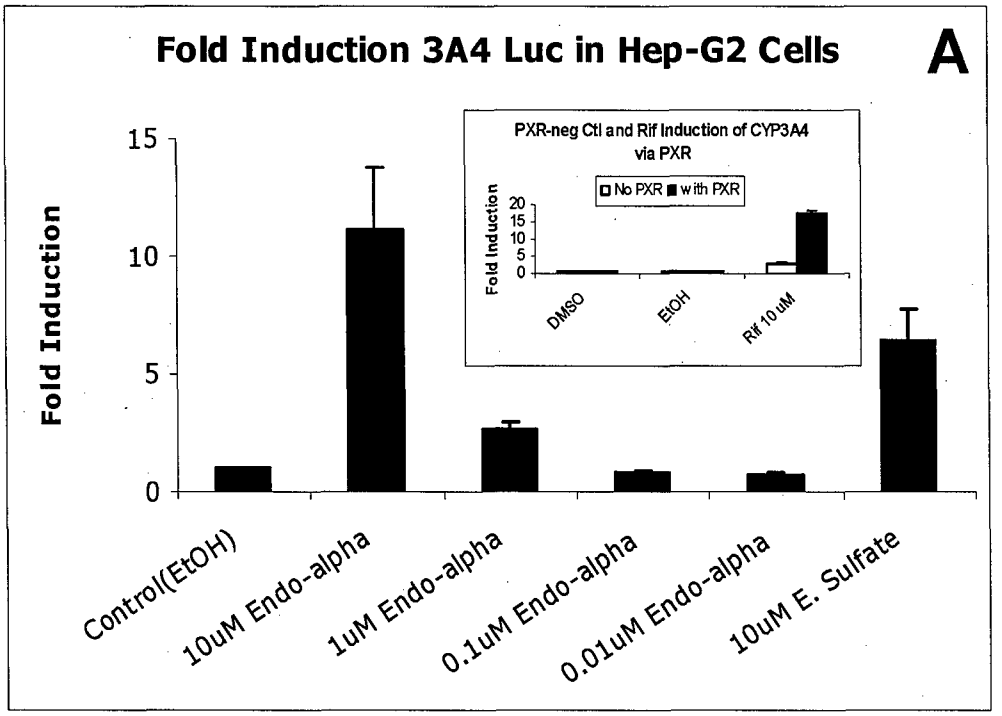

A: Adenylate Kinase Activity in Human Hepatoma (HepG2) Cells
24 h
48 h
72 h
Relative Luminescence Unit, Mean ± SE, n=6
3.5e+5
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0
Control
3.125 uM
6.25 uM
12.5 uM
25 uM
50 uM
100 uM
B: Adenylate Kinase Activity in Human Hepatic Cells
24 h
48 h
72 h
Relative Luminescence Unit, Mean ± SE, n=2/4
3.5e+5
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0
Control
1 uM
6.25 uM
12.5 uM
25 uM
50 uM
100 uM
Concentration of Endosulfan
C: Caspase-3/7 Activity in Human Hepatoma (HepG2) Cells
24 h
48 h
72 h
Relative Luminescence Units, Mean±SE, n=6
5e+5
4e+5
3e+5
2e+5
1e+5
0
Control
1.0 uM
3.125 uM
6.25 uM
12.5 uM
25 uM
50 uM
100 uM
D: Caspase-3/7 Activity in Human Hepatic Cells
24 hr
48 hr
72 hr
Relative Luminescence Unit, Representative data
Bar = Mean of 2 individuals / 4 wells
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
Control
1 uM
6.25 uM
50 uM
100 uM
100uM+0.05uM
Endosulfan and Z-DEVD-FMK concentrations

Fig. 3


Fold Induction 3A4 Luc in Hep-G2 Cells
A
Fold Induction
15
10
5
0
Control(EtOH)
10uM Endo-alpha
1uM Endo-alpha
0.1uM Endo-alpha
0.01uM Endo-alpha
10uM E. Sulfate
PXR-neg Ctl and Rif Induction of CYP3A4 via PXR
No PXR
with PXR
Fold Induction
20
15
10
5
0
DMSO
EtOH
Rif 10 uM

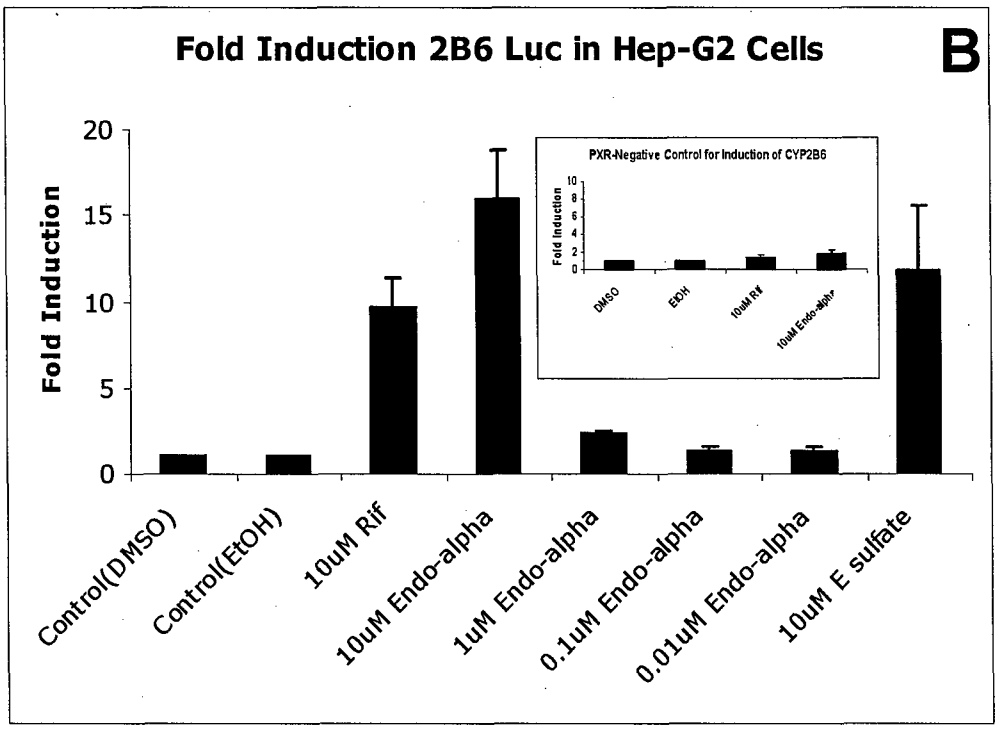

Fold Induction 2B6 Luc in Hep-G2 Cells
B
Fold Induction
20
15
10
5
0
Control(DMSO)
Control(EtOH)
10uM Rif
10uM Endo-alpha
1uM Endo-alpha
0.1uM Endo-alpha
0.01uM Endo-alpha
10uM E sulfate
PXR-Negative Control for Induction of CYP2B6
Fold Induction
10
8
6
4
2
0
DMSO
EtOH
10uM Rif
10uM Endo-alpha

Fig. 4

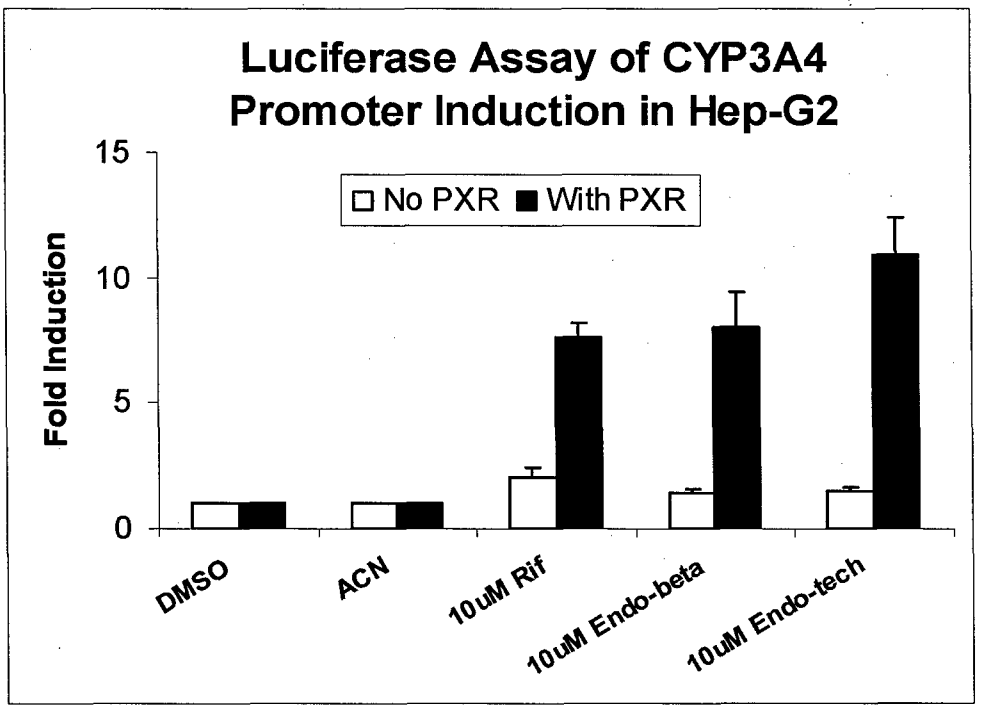

Luciferase Assay of CYP3A4 Promoter Induction in Hep-G2
No PXR
With PXR
Fold Induction
15
10
5
0
DMSO
ACN
10uM Rif
10uM Endo-beta
10uM Endo-tech

Fig 5.

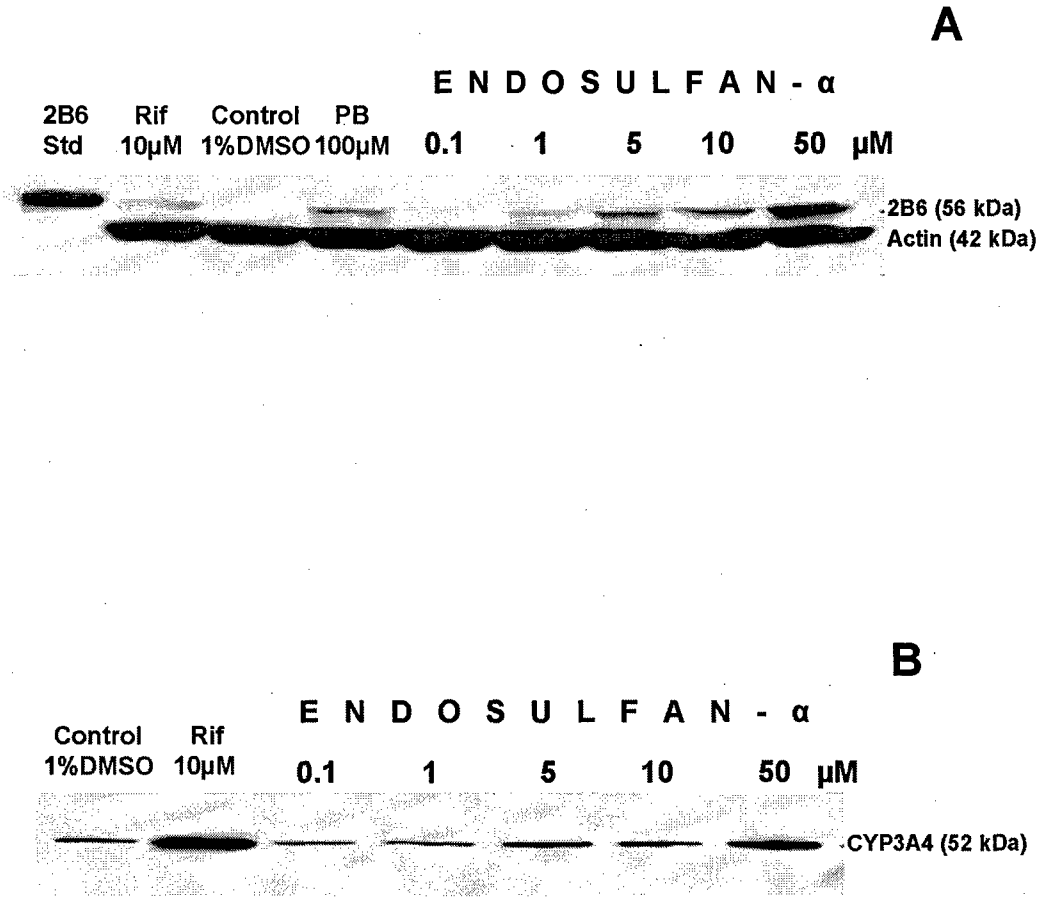

A
ENDOSULFAN - α
2B6 Std
Rif 10μM
Control 1%DMSO
PB 100μM
0.1
1
5
10
50
μM
2B6 (56 kDa)
Actin (42 kDa)
B
ENDOSULFAN - α
Control 1%DMSO
Rif 10μM
0.1
1
5
10
50
μM
CYP3A4 (52 kDa)

Fig. 6

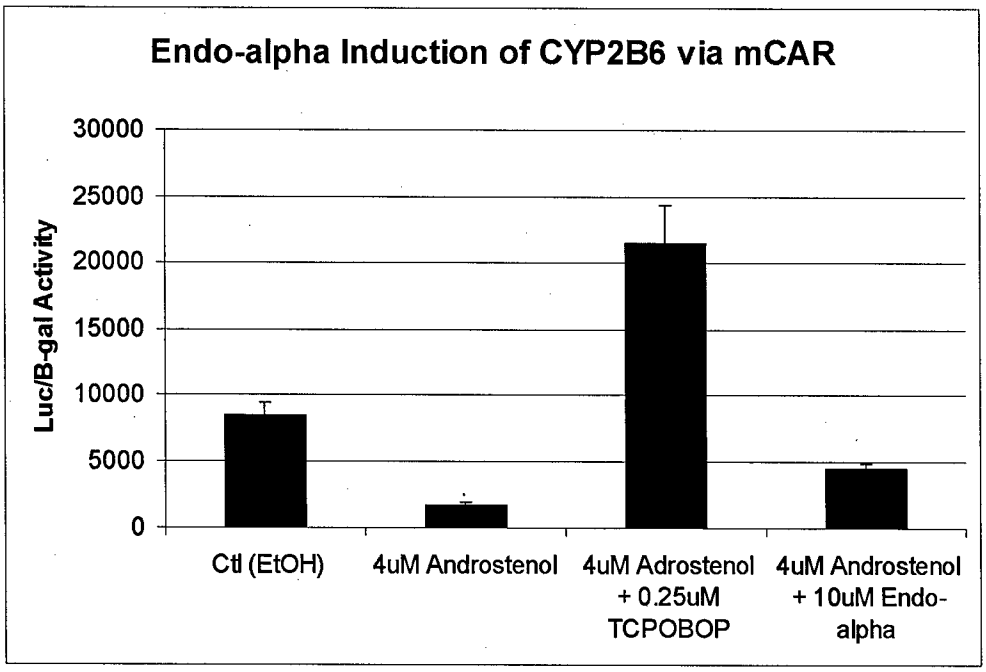

Endo-alpha Induction of CYP2B6 via mCAR
Luc/B-gal Activity
30000
25000
20000
15000
10000
5000
0
Ctl (EtOH)
4uM Androstenol
4uM Adrostenol + 0.25uM TCPOBOP
4uM Androstenol + 10uM Endo-alpha

Fig 7.

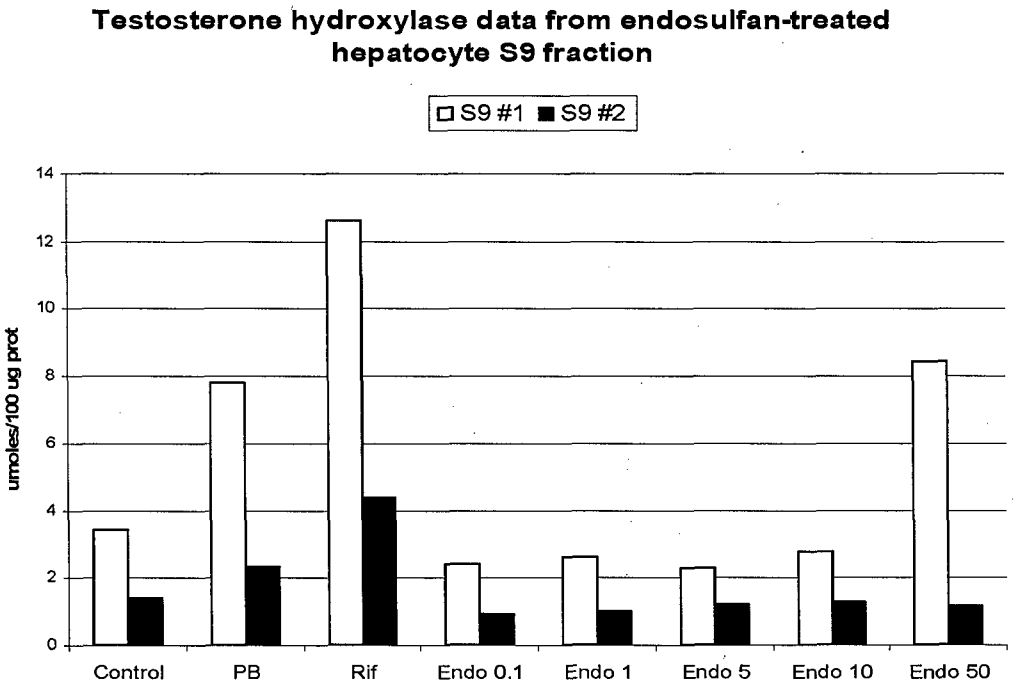
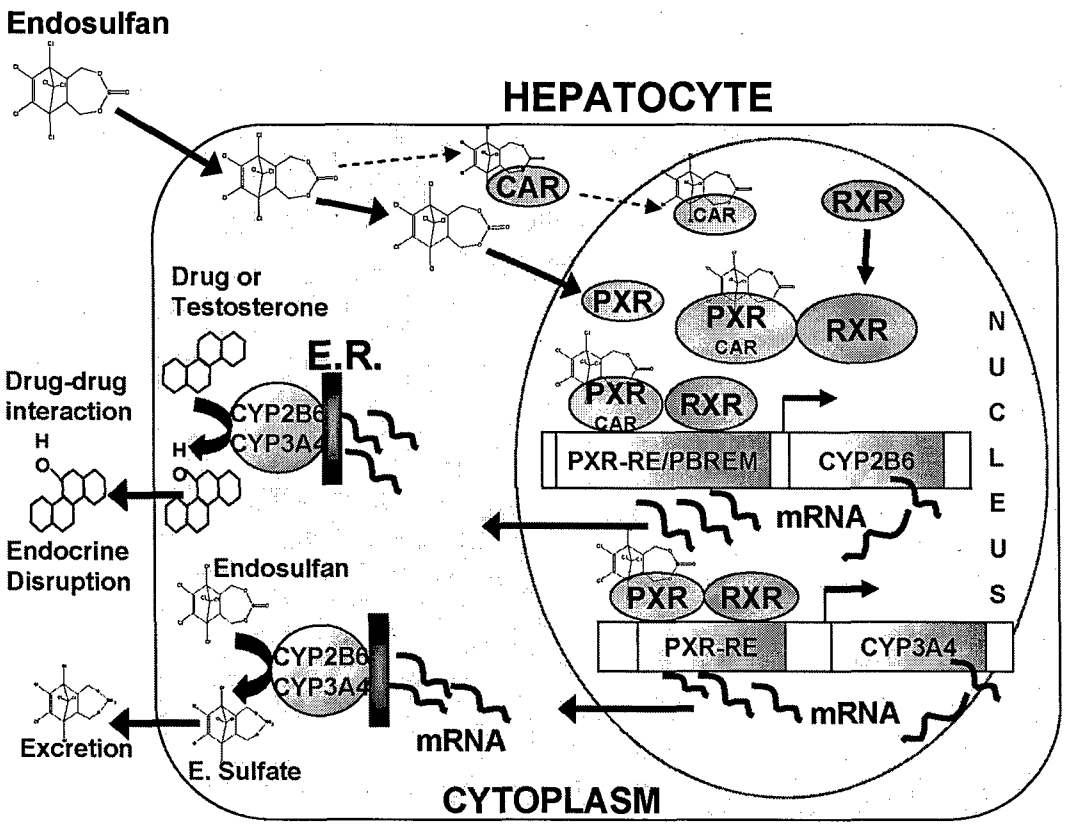

Testosterone hydroxylase data from endosulfan-treated hepatocyte S9 fraction
S9 #1
S9 #2
umoles/100 ug prot
0
2
4
6
8
10
12
14
Control
PB
Rif
Endo 0.1
Endo 1
Endo 5
Endo 10
Endo 50

Fig. 8.


Endosulfan
HEPATOCYTE
CAR
CAR
RXR
Drug or
Testosterone
PXR
PXR
CAR
RXR
E.R.
PXR
CAR
RXR
Drug-drug
interaction
H
CYP2B6
CYP3A4
H
PXR-RE/PBREM
CYP2B6
NUCLEUS
mRNA
Endocrine
Disruption
Endosulfan
PXR
RXR
PXR-RE
CYP3A4
CYP2B6
CYP3A4
mRNA
mRNA
Excretion
E. Sulfate
CYTOPLASM